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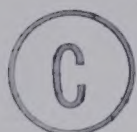
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STUDIES ON THE MECHANISM OF PORPHYRIA-INDUCTION BY DRUGS
AND THE NATURE OF THE INHIBITION BY PROTOHEMIN
OF DRUG-INDUCED PORPHYRIA

by



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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

FALL, 1969

Thesis
1969(F)
64D

ABSTRACT

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

A series of compounds has been tested for porphyria-inducing
The undersigned certify that they have read, and recommend to the Faculty
of Graduate Studies for acceptance, a thesis entitled "Studies on the
Mechanism of Porphyria-Induction by Drugs and the Nature of the Inhibition
by Protohemin of Drug-Induced Porphyria," submitted by Dr. Dennis Wayne
Schneck in partial fulfilment of the requirements for the degree of Doctor
of Philosophy. A series of aliphatic di-esters, aliphatic monoamides, and

ABSTRACT

A series of compounds has been tested for porphyria-inducing activity in chick embryo liver cells. The results obtained suggested that the critical feature for activity in a series of aliphatic and aromatic compounds studied might be an ester or amide group which is sterically hindered from hydrolysis. To test this idea studies were conducted of the hydrolytic activity of an esterase and amidase prepared from chick embryo liver towards a series of aliphatic di-esters, aliphatic monoamides, and aromatic amides. The results obtained with aliphatic di-esters revealed that the most active porphyria-inducing drug was hydrolyzed with the greatest difficulty. These observations provide support for the idea that a sterically hindered ester group is necessary for activity. The results obtained with aliphatic amides showed that in general the order of increasing porphyria-inducing activity paralleled the order of increasing difficulty to enzymic hydrolysis. These observations supported the idea that a sterically hindered amide group is necessary for activity. The results obtained with the aromatic amides showed no correlation between porphyria-inducing activity and the difficulty of enzymic hydrolysis. Our results lead us to conclude that a sterically hindered ester group is required for porphyria-inducing activity but many potent porphyria-inducing amides exist in which there is no steric hindrance to hydrolysis.

Important features required for porphyria-inducing activity in the griseofulvin molecule were revealed by testing a series of griseofulvin analogues.

Using chick embryo liver cells it has been shown that the action

of all porphyria-inducing drugs could be inhibited by protohemin. These observations supported current ideas on the mechanism of action of porphyria-inducing drugs. The kinetic nature of protohemin inhibition of porphyria-induction by allylisopropylacetamide (AIA) was studied. Inhibition was noted when minute doses of protohemin were employed but porphyria-induction by AIA could not be completely abolished when high doses of protohemin were used. The inhibition by protohemin was shown to be non-competitive in nature, an observation which is not in accord with current ideas on the mechanism of protohemin inhibition of porphyria-induction.

Incubation of cells for 10 hours with AIA (first incubation period) followed by removal of AIA leads on re-incubation (second incubation period) to an increase in porphyrin accumulation. If protohemin or puromycin are present during the second incubation period, increased porphyrin accumulation was not observed. On the other hand if actinomycin D is present during the second incubation period porphyrin accumulation was not affected. These results showed that protohemin inhibition of porphyria-induction is not limited to inhibition of transcription as has been believed hitherto. The results obtained in these and the kinetic experiments indicate that protohemin inhibits the translation of m-RNA to δ -aminolevulinic acid synthetase, δ -aminolevulinic acid synthetase itself, or both. The inhibition by protohemin of porphyrin accumulation in response to a drug is thus not confined to the transcription process as thought hitherto.

ACKNOWLEDGMENTS

It was my good fortune to be a student of Dr. G. S. Marks whose enthusiasm and keen mind were of invaluable assistance throughout the course of this study.

This study was made possible through the patience and encouragement of my wife, Saralee. To express my thanks I dedicate this thesis to her.

I would like to thank the Medical Research Council for providing financial support throughout the course of this study.

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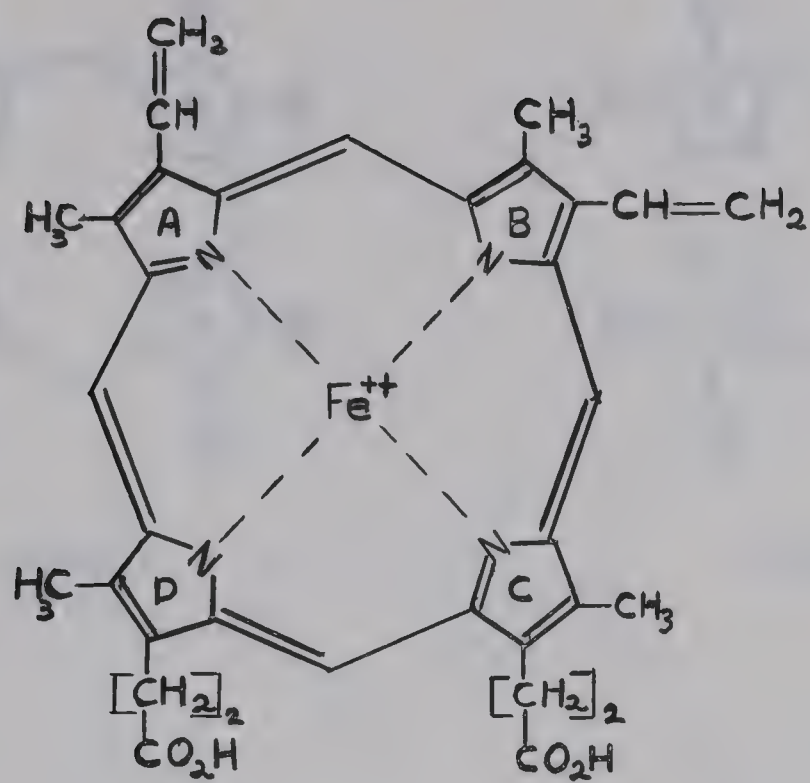
CHAPTER I GENERAL INTRODUCTION

A. Porphyrin Chemistry

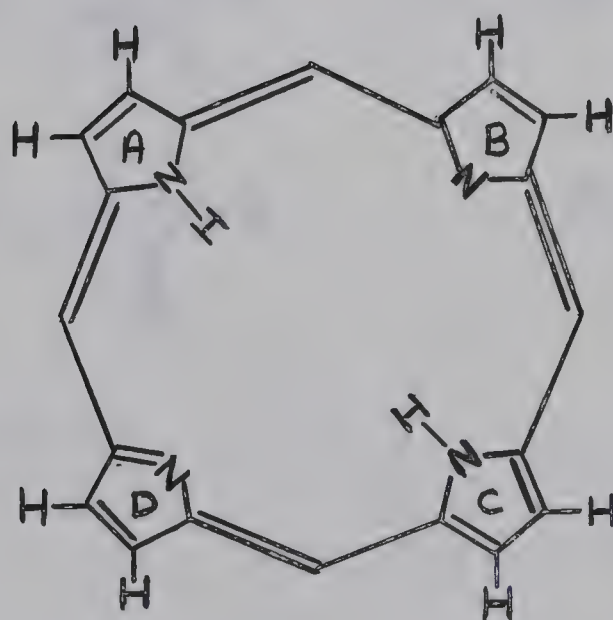
Protoheme (Fig. 1A), the prosthetic group of the red blood pigment hemoglobin, is a derivative of porphin (Fig. 1B) which consists of four pyrrole rings attached to each other by four methine bridge carbons. Removal of the iron atom from protoheme yields protoporphyrin IX. The uro- and coproporphyrins are derivatives of porphin in which each of the four pyrrole rings bears two different substituents A and B. This arrangement of substituents leads to the existence of four different isomers (Fig. 2). The position is more complicated in the case of proto- and mesoporphyrin (Fig. 3) where there are two substituents, A and B in rings A and B, and two substituents, A and C in rings C and D. This arrangement of substituents leads to the existence of fifteen isomers and the porphyrin derived by removing iron from protoheme has an arrangement of substituents leading to its assignment as the ninth isomer of the series. For this reason it is designated protoporphyrin IX (Marks, 1969).

Because of the conjugated double bond system these substances are planar and have characteristic absorption spectra. On irradiation with light at about 400 mμ porphyrins have a characteristic very intense orange to red fluorescence. This fluorescence can be detected by eye at concentrations of the order of 10^{-8} M and photoelectrically down to 10^{-10} M (Phillips, 1963). The fluorometric determination of porphyrin fluorescence is a method by which minute amounts of porphyrin can be detected. Metal cations co-ordinate with porphyrins to form square planar tetra-coordinate complexes. Protoheme is the tetra-coordinate complex of protoporphyrin IX and ferrous ion (Falk, 1963).

Treatment of uro- and coproporphyrin with reducing agents such

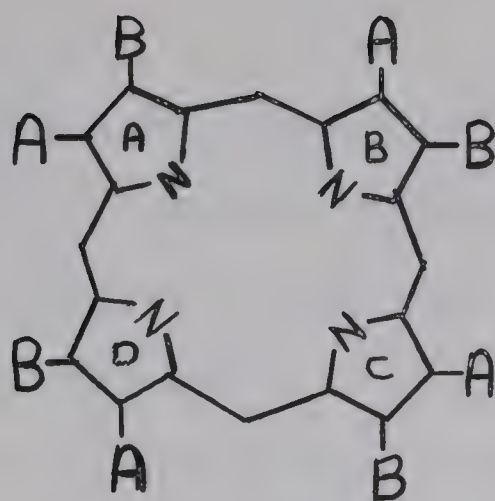


(A)

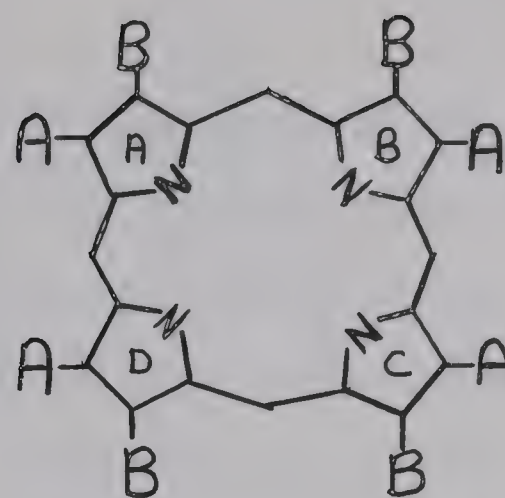


(B)

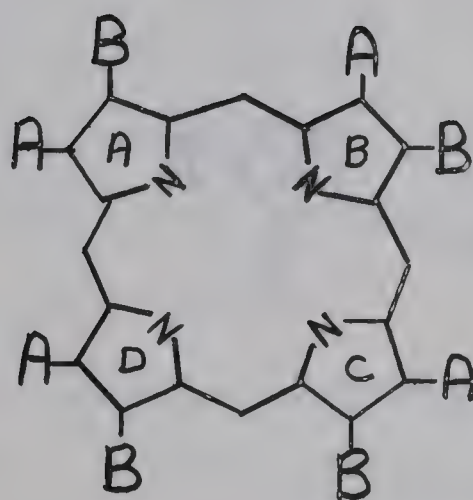
Fig. 1 Structures of Protoheme (A) and Porphin (B).



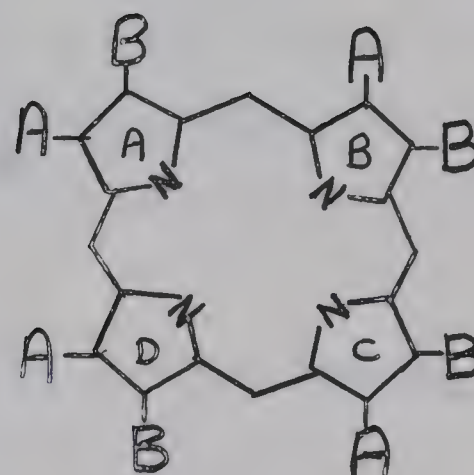
Isomer I



Isomer II



Isomer III

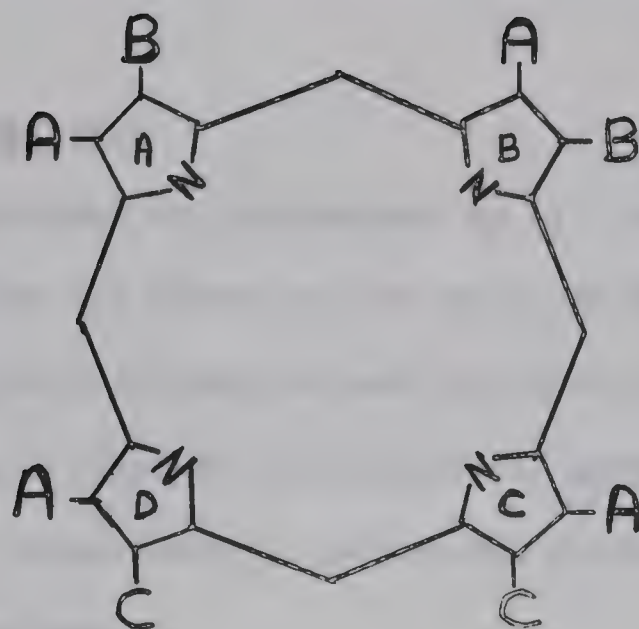


Isomer IV

for Copro- $A = \text{CH}_3$, $B = \text{CH}_2\text{CH}_2\text{CO}_2\text{H}$

for Uro- $A = \text{CH}_3\text{CO}_2\text{H}$, $B = \text{CH}_2\text{CH}_2\text{CO}_2\text{H}$

Fig. 2 Diagrammatic Representation of Isomers of
Uro- and Coproporphyrin



for Meso- $A = \text{CH}_3$, $B = \text{C}_2\text{H}_5$, $C = \text{CH}_2\text{CH}_2\text{COOH}$

for Proto- $A = \text{CH}_3$, $B = \text{CH}=\text{CH}_2$, $C = \text{CH}_2\text{CH}_2\text{COOH}$

Fig. 3 Diagrammatic Representation of Meso- and
Protoporphyrin IX

as sodium amalgam results in the addition of six hydrogen atoms to the porphyrin. These hexahydro- derivatives known as porphyrinogens (Fig. 4E & 4F) are of biochemical importance since they are intermediates in the biosynthesis of protoheme. The reduction destroys conjugation and consequently these molecules are colorless and non-fluorescent (Marks, 1969).

B. Protoheme Biosynthesis

While protoheme is synthesized by all cells of the body, erythropoietic tissue and liver are the major sites of synthesis. In erythropoietic tissue protoheme is used for hemoglobin synthesis whereas in the liver it is utilized as the prosthetic group of a variety of hemoproteins, viz., cytochrome C, cytochrome P-450, peroxidase, catalase, and tryptophan pyrrolase.

In recent years the biosynthesis of protoheme has been elucidated primarily as a result of work conducted by Shemin and Granick in the United States and Neuberger and Rimington in Great Britain (De Matteis, 1967). The first step in the biosynthesis of protoheme is the condensation of one molecule of succinyl-CoA (Fig. 4A) with one molecule of glycine (Fig. 4B) by the enzyme δ -aminolevulinic acid synthetase (Shemin, 1956; Kikuchi et al., 1958; Gibson et al., 1958) to form δ -aminolevulinic acid (δ -ALA, Fig. 4C). Pyridoxal phosphate participates as a cofactor in this reaction (Lascelles, 1957; Schulman & Richert, 1957). Succinyl-CoA is derived mainly via oxidation of α -ketoglutarate through operation of the tricarboxylic acid cycle although some is generated directly from succinate and CoA by succinyl-CoA synthetase in the presence of ATP (Brown, 1958; Granick & Urata, 1963; Shemin & Kumin,

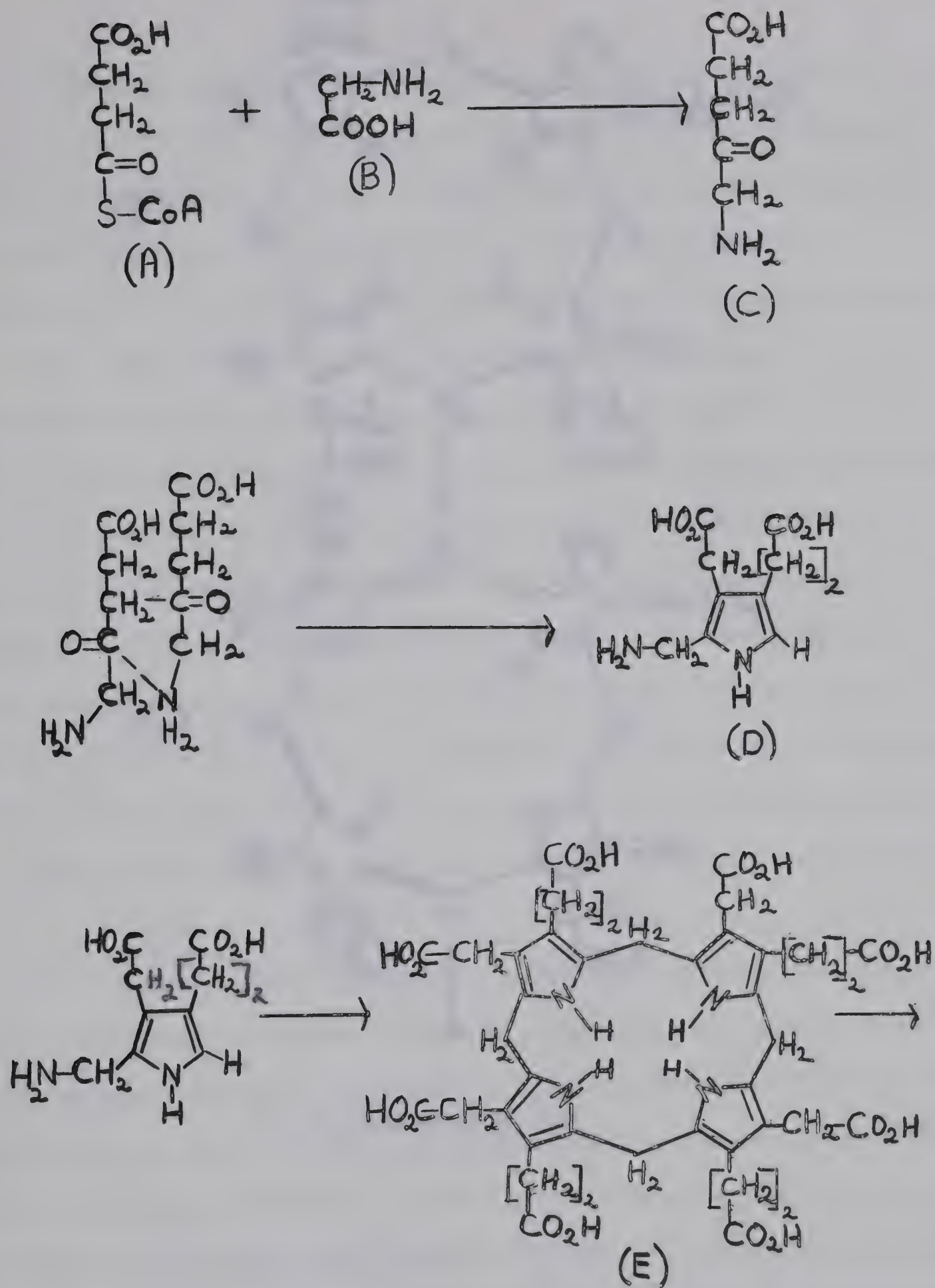


Fig. 4 Biosynthesis of Protoheme

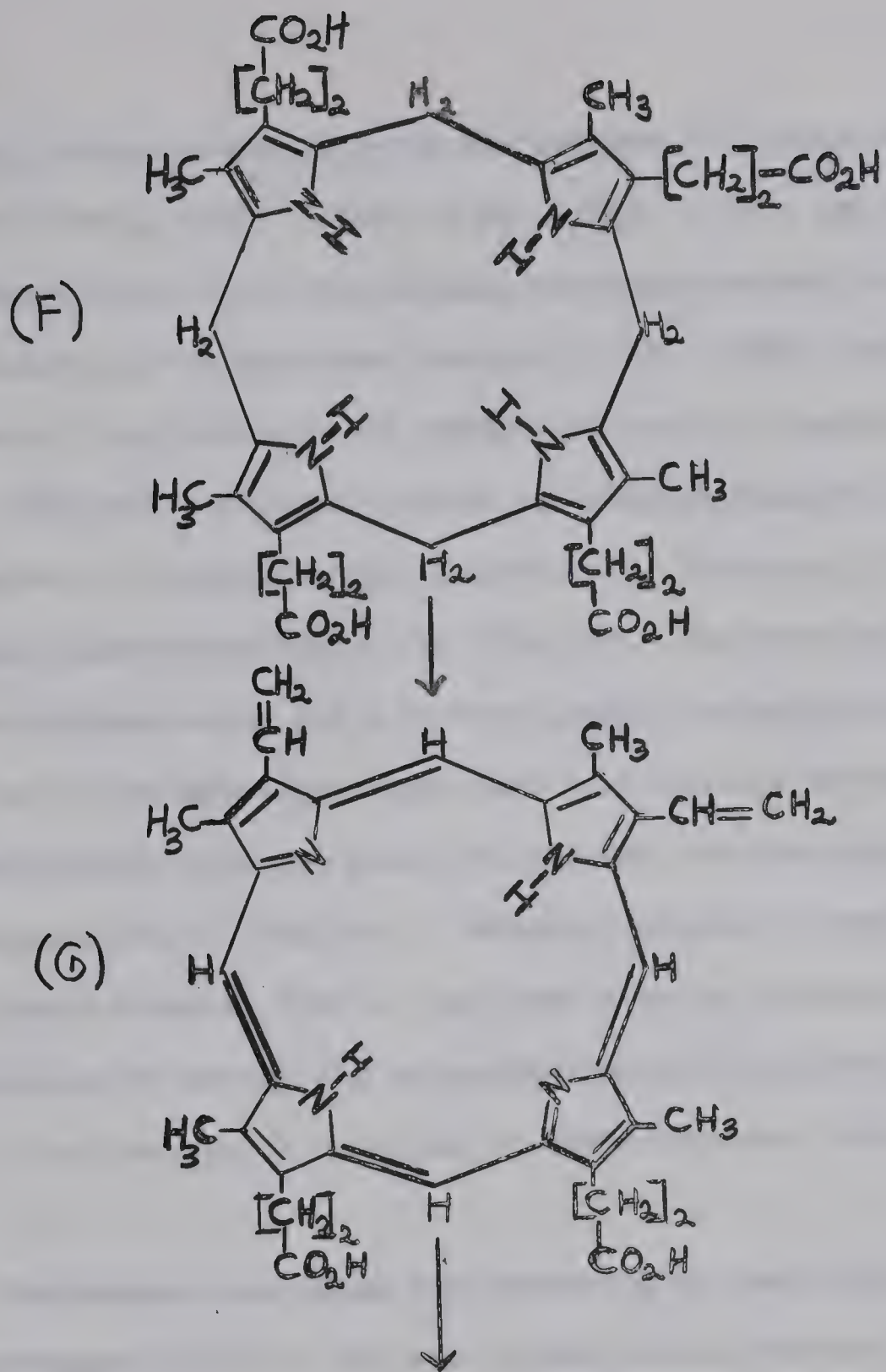


Fig. 4 (Continued) Biosynthesis of Protoheme

1952). Next, two molecules of δ -ALA are combined by δ -aminolevulinic acid dehydratase (Shemin, 1956; Gibson et al., 1955) to form the monopyrrole porphobilinogen (Fig. 4D). Two enzymes, uroporphyrinogen I-synthetase and uroporphyrinogen III-cosynthetase (Bogorad, 1958a; 1958b) catalyze the condensation of four molecules of porphobilinogen to uroporphyrinogen III (Fig. 4E). The acetic acid side chains of uroporphyrinogen III are decarboxylated by uroporphyrinogen decarboxylase (Mauzerall & Granick, 1958) to form coproporphyrinogen III (Fig. 4F). Coproporphyrinogen oxidase removes two hydrogen atoms and a carboxyl group from each of the two propionic acid side chains in rings A and B of coproporphyrinogen III and at the same time six hydrogen atoms are removed from the ring system to yield protoporphyrin IX (Fig. 4G). Molecular oxygen is required for this reaction (Sano & Granick, 1961). The final step in protoheme biosynthesis is the chelation of ferrous ion by protoporphyrin IX to form protoheme (Fig. 4H). The reaction is catalyzed by ferrochelatase (Labbe & Hubbard, 1960).

The enzymes catalyzing the conversion of δ -ALA (Fig. 4C) to coproporphyrinogen III (Fig. 4F) are in the soluble fraction of animal cells whereas δ -ALA synthetase and the enzymes converting coproporphyrinogen III to protoheme (Fig. 1A) are located in mitochondria (Sano & Granick, 1961). δ -ALA must therefore be released from the mitochondria before becoming available for porphyrinogen synthesis. On the other hand coproporphyrinogen III must move into mitochondria from the soluble fraction before being converted to protoheme.

C. Human and Experimental Porphyria

The clarification of the protoheme biosynthetic pathway provided the background for the study of a group of disorders in which inborn or acquired derangements of enzymes involved in protoheme biosynthesis result in an increased excretion of porphyrins, their precursors, or both in the excreta. These disorders have been designated porphyrias. The studies of Schmid et al. (1954) revealed that in some cases of porphyria the porphyrins accumulated in the bone marrow while in other cases porphyrins and porphyrin precursors accumulated in the liver. For this reason the disorders of porphyrin metabolism have been classified as erythropoietic or hepatic porphyria. The hepatic porphyrias are subdivided into three major types, viz., acute intermittent porphyria (Swedish type), cutaneous hepatic porphyria (South African type), and acquired hepatic porphyria (Goldberg & Rimington, 1962). In the hepatic porphyrias the defect appears to be limited to liver cells.

Acute intermittent porphyria is characterized by the dominance of gastrointestinal and neurologic symptoms and the absence of skin photosensitivity (Goldberg & Rimington, 1962). In the acute phase of the disease large amounts of the porphyrin precursors, δ -ALA and porphobilinogen, are excreted in the urine. In the latent phase of the disease or during remission the urinary excretion of these precursors may be within normal limits or slightly elevated. Among the factors believed responsible for precipitating the clinical manifestations of the disease, the administration of various drugs is the most important.

Cutaneous hepatic porphyria is characterized by gastrointestinal and neurologic symptoms but differs clinically from acute intermittent

porphyria by the presence of skin photosensitivity. During the acute phase of the disease excessive amounts of δ -ALA and porphobilinogen are excreted in the urine. This abnormal excretion may return to normal during remission. Unlike acute intermittent porphyria, a high fecal excretion of copro- and protoporphyrin is noted during remission and during the acute phase. Administration of drugs may also precipitate the clinical manifestations of the disease.

An acquired, non-hereditary form of hepatic porphyria has been described. In 1956 thousands of Turkish peasants developed severe photosensitivity following consumption of seed-wheat treated with the fungicide hexachlorobenzene. In addition, a high excretion of uro- and coproporphyrin in the urine was noted. Cam (1959) suggested the etiology of the porphyria and this suggestion was supported by the finding that hexachlorobenzene readily caused a porphyria when fed to rats, guinea pigs, mice and rabbits (Ochner & Schmid, 1961; De Matteis et al., 1961).

Since the introduction of barbiturates into clinical medicine in 1903 there has arisen the suspicion that relatively small amounts of these drugs precipitate acute and--at times--fatal attacks of clinically latent and asymptomatic hereditary hepatic porphyria (Waldenstrom, 1957). Recently a variety of drugs have been demonstrated to act in a similar fashion to the barbiturates; these include griseofulvin, meprobamate (Miltown), chloradiazepoxide (Librium), 5,5-diphenylhydantoin, and N-2-dimethyl-2-phenylacetamide (Cowger & Labbe, 1965). While small doses of these drugs can induce an attack of porphyria in patients carrying the inherited trait of hepatic porphyria, much larger doses of these drugs will cause a disordered porphyrin metabolism in normal animals (Goldberg

& Rimington, 1962; Schmid, 1960). The disorders of porphyrin metabolism induced in normal animals by drug administration are referred to as experimental porphyrias. Although there is a striking similarity in the biochemical abnormality observed in the experimental and in the hepatic porphyrias, the clinical manifestations observed in the hepatic porphyrias are not reproduced in the experimental porphyrias. For this reason caution is required in extrapolating the data from the experimental animal porphyrias to the inherited disease. Nevertheless, valuable information regarding the inherited disease has been obtained from studies of the experimental porphyrias.

With the steps of porphyrin biosynthesis clarified it was possible for Granick & Urata (1963) to study the metabolic defect occurring in experimental porphyria. The administration of the potent porphyria-inducing compound 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC, Fig. 5A) to guinea pigs produced a disturbance in porphyrin metabolism similar to that in acute intermittent porphyria.

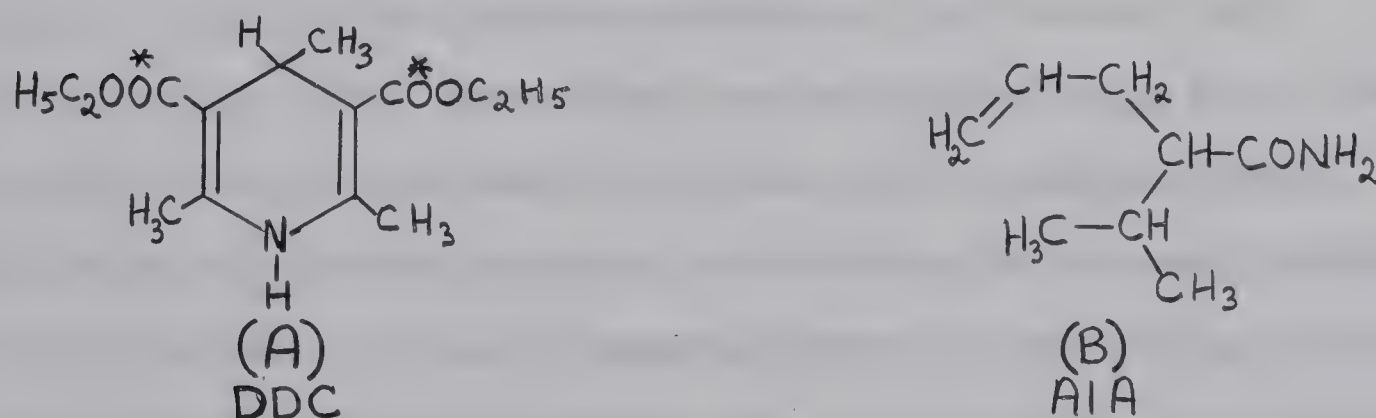


Fig. 5

The activity of δ -ALA synthetase, the first enzyme in the protoheme biosynthetic pathway, was found to be increased more than forty

fold. The activities of the other enzymes in the pathway showed no significant change as compared to controls. Incubation of mitochondria isolated from normal guinea pig liver with DDC did not result in an enhancement of δ -ALA synthetase activity suggesting that DDC either increased the synthesis of δ -ALA synthetase or reduced its rate of destruction or both.

Granick (1963; 1966) developed a technique of primary cell culture to study the mechanism of porphyria induction in liver cells derived from chick embryos. Exposure of these cells to porphyria-inducing drugs resulted in a large accumulation of porphyrins which could be visualized by the intense fluorescence when observed with a fluorescence microscope. Granick assumed that the intensity of porphyrin fluorescence was proportional to the activity of δ -ALA synthetase. When mitomycin C, an inactivator of DNA (Reich et al., 1961), was incubated simultaneously with the potent porphyria-inducing drug allylisopropylacetamide (AIA, Fig. 5B) porphyrin accumulation was abolished. Actinomycin D, an inhibitor of m-RNA synthesis (Reich et al., 1961; Hurwitz et al., 1962), and puromycin, an inhibitor of protein synthesis at the ribosomal level (Nathans & Lipman, 1961), also blocked porphyria induction by AIA. From these results Granick concluded that AIA and other porphyria-inducing drugs produce experimental porphyria by stimulating an increased synthesis of δ -ALA synthetase. AIA was subsequently shown to cause an eight fold increase in the activity of the above enzyme in chick embryo liver (Granick, 1966). Granick (1966) demonstrated that protohemin (Fig. 6) inhibited porphyria induction by AIA and assumed that protoheme (Fig. 1A) would have the same effect. It is worth noting that protohemin was utilized because

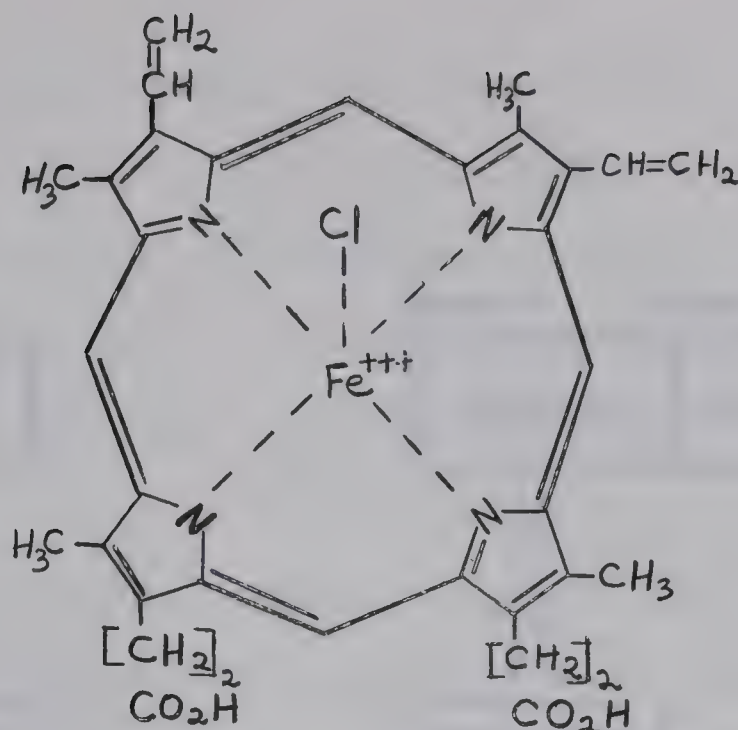


Fig. 6. Protohemin

of the difficulty of maintaining protoheme in the reduced state. Since protohemin did not interfere with the conversion of δ -ALA (Fig. 4C) to porphyrins and did not alter the δ -ALA synthetase activity of guinea pig liver mitochondria Granick suggested that protoheme functions as a co-repressor for δ -ALA synthetase as described below.

In the mechanism controlling enzyme formation proposed by Jacob, Monod, and Wollman (1962) a specific repressor molecule inhibits a specific DNA region (operon) from being transcribed into m-RNA as illustrated in Fig. 7. Granick (1966) considers the active repressor of the operon coding for δ -ALA synthetase to be a protein apo-repressor in combination with protoheme. He suggests that the porphyria-inducing drugs compete with and displace protoheme from the aporepressor. The resulting drug-aporepressor complex is considered unable to inhibit operon activity

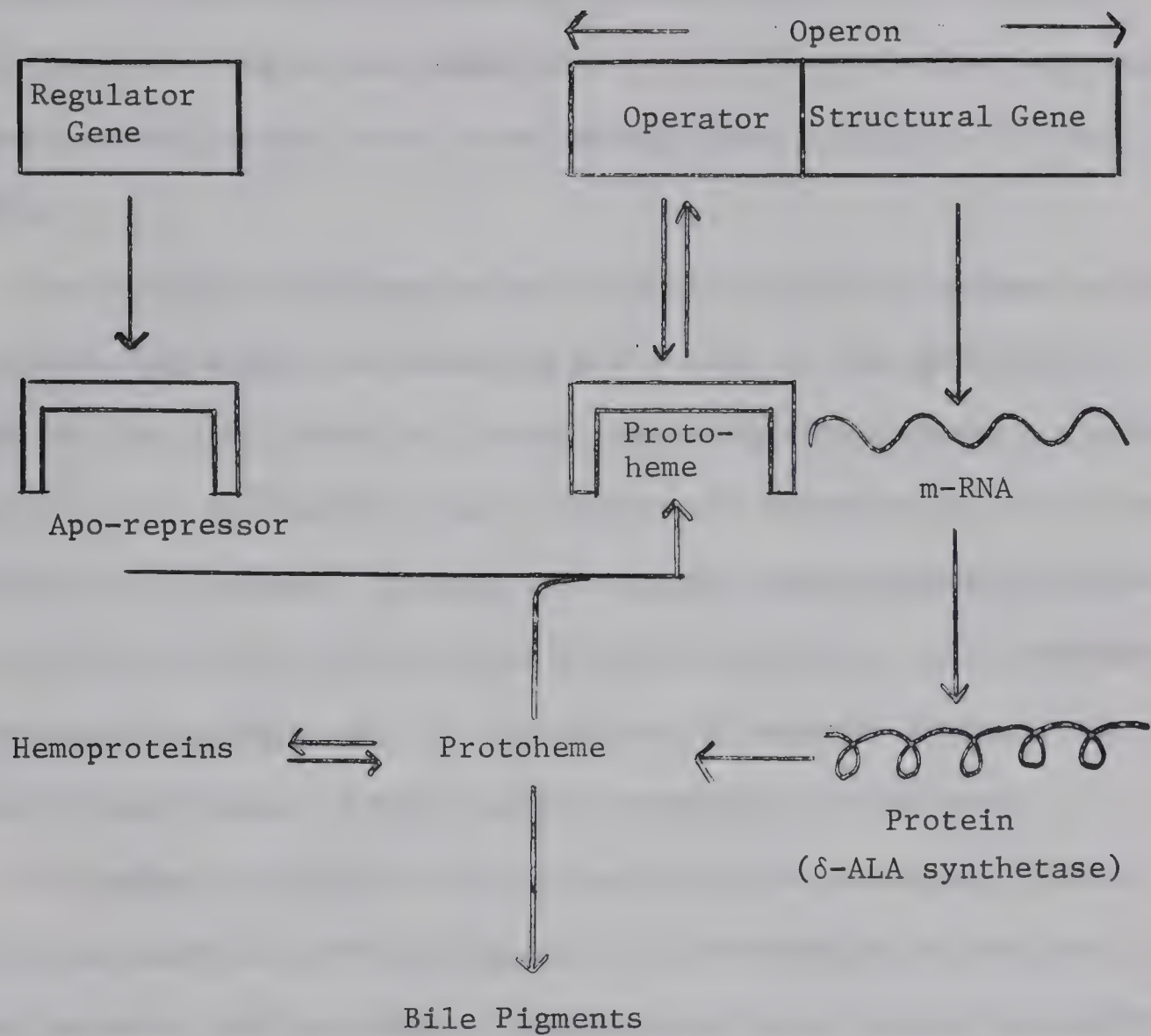


Fig. 7 The Operon Concept Applied to the Regulation of the Synthesis of δ -ALA Synthetase

so that increased m-RNA synthesis takes place and as a result more δ -ALA synthetase is made. This increased enzyme synthesis is responsible for the biochemical disturbance in experimental porphyria. It is of considerable interest that an increased level of δ -ALA synthetase has recently been observed in a patient with acute intermittent porphyria (Tschudy et al. 1965).

According to the hypothesis of Granick (1966) protoheme and the porphyria-inducing drugs are competing for a site on the aporepressor. This view implies that there is a structural resemblance among porphyria-inducing drugs and furthermore that a structural resemblance exists between these drugs and protoheme. In order to explore this hypothesis further we have: (1) carried out a study of the structure-activity relationships of porphyria-inducing drugs, and (2) carried out a detailed study of the inhibition by protohemin of drug-induced porphyrin biosynthesis.

In order to simplify the following discussion we shall deal first with our study of structure-activity relationships in section (D) below and secondly with protohemin inhibition of drug-induced porphyrin biosynthesis in the following section (E).

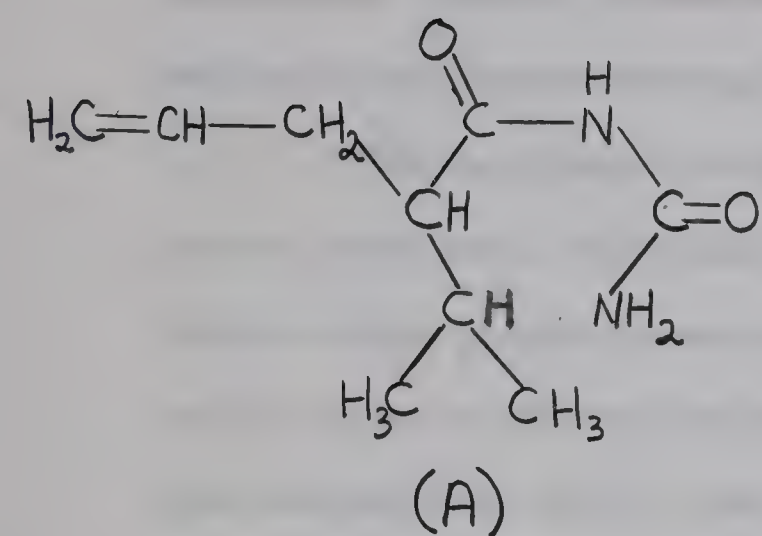
D. Structure-Activity Relationships

Small amounts of some of the barbiturates can precipitate acute, and--at times--fatal attacks of clinically latent hepatic porphyria. Goldberg and Rimington (1962) have investigated the relationship between chemical structure and porphyria-inducing activity in this group of compounds. They concluded that the structural features required for porphyria-inducing activity in rabbits were one allyl group together with an acid

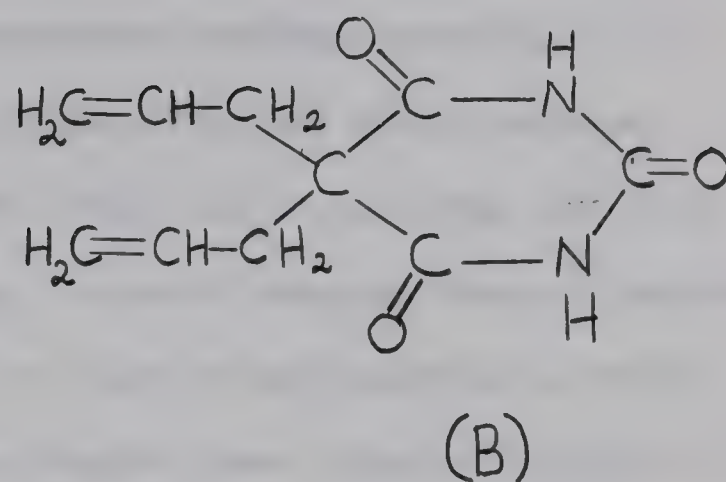
amide as in allylisopropylacetamide (Fig. 5B) or a ureide as in 2-isopropyl-pent-4-enoylurea (Fig. 8A) or a cyclic ureide as in 5,5-diallylbarbituric acid (Fig. 8B). These results were confirmed by Stich & Decker (1955) and by Talman et al. (1957). Further studies revealed that a large number of drugs, which were structurally unrelated to the barbiturate group, such as DDC (Fig. 5A) (Solomon & Figge, 1959) and griseofulvin (Fig. 8C) (Cowger, 1965; Granick, 1966) were potent porphyria-inducing drugs so that the results reached by earlier workers were clearly inadequate in defining structural features required for activity.

According to the hypothesis of Granick (1966) porphyria-inducing drugs compete with protoheme for a site on the aporepressor leading to increased formation of protoheme which is utilized as the prosthetic group of cytochrome P-450. The cytochrome P-450 formed is thought to participate in the oxidative metabolism of the drug. It therefore follows from this hypothesis that all porphyria-inducing drugs should be oxidatively metabolized. Recently Hirsch et al. (1967) have suggested that the underlying critical feature for activity in the AIA (Fig. 5B) and DDC (Fig. 5A) series of compounds is an ester or amide group which is sterically hindered from hydrolysis. It was further suggested by these workers that drugs which cannot be metabolized by a hydrolytic mechanism because of steric factors are oxidatively metabolized and increased protoheme formation is therefore required.

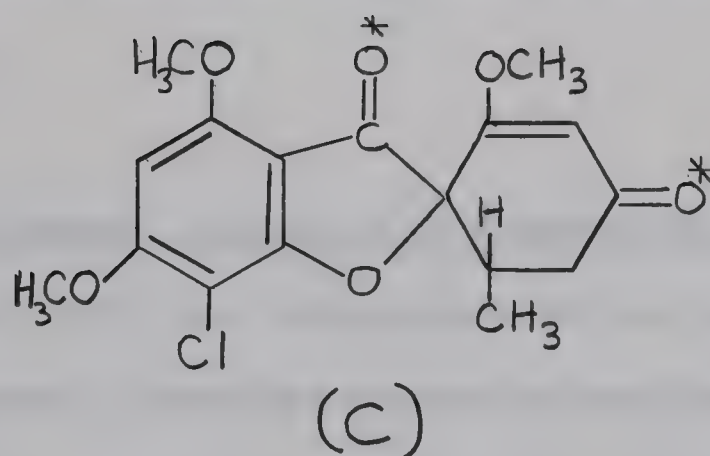
In chapter II sections A & B, we have investigated the validity of the idea that the underlying critical feature for activity in the AIA and DDC groups of compounds is an ester or amide group which is sterically



2-Isopropylpent-4-enoylurea



5,5-Diallylbarbituric acid



Griseofulvin

Fig. 8 Structures of 2-Isopropylpent-4-enoylurea (A), 5,5-Diallylbarbituric acid (B), and Griseofulvin (C)

hindered from hydrolysis. This was done by selecting a series of compounds with varying degrees of steric hindrance to the hydrolysis of ester and amide groups and testing their porphyria-inducing activity.

The antifungal drug griseofulvin (Fig. 8C) which bears no obvious structural relationship to DDC (Fig. 5A) or AIA (Fig. 5B) is a highly active porphyria-inducing drug. Granick compared Courtald models of DDC and griseofulvin and pointed out that it was possible to twist a Courtald model of DDC so that the two oxygen atoms (starred in Fig. 5A) were separated by the same distance as the two keto-oxygen atoms in griseofulvin (starred in Fig. 8C) and he suggested that this inter-oxygen distance was necessary for optimal porphyria-inducing activity. Hirsch (1966) has carried out preliminary studies to test this hypothesis. We have extended these studies by testing the porphyria-inducing activity of several griseofulvin analogues. The results of these studies are reported in Chapter II, C.

E. Nature of Protohemin Inhibition of Porphyrin Biosynthesis

Granick (1966) has demonstrated in the chick embryo liver cell system that protohemin inhibited porphyria-induction by AIA. Since the degree of inhibition seemed to depend on the relative concentrations of protohemin and AIA it was suggested that the inhibition was of a competitive type. Since the scheme formulated in Fig. 7 is based on the idea of competition between porphyria-inducing drugs and protohemin for a site on the aporepressor the following facts should be demonstrable for the hypothesis to have any possible validity: (1) Inhibition by protohemin of porphyria-induction by all porphyria-inducing drugs.

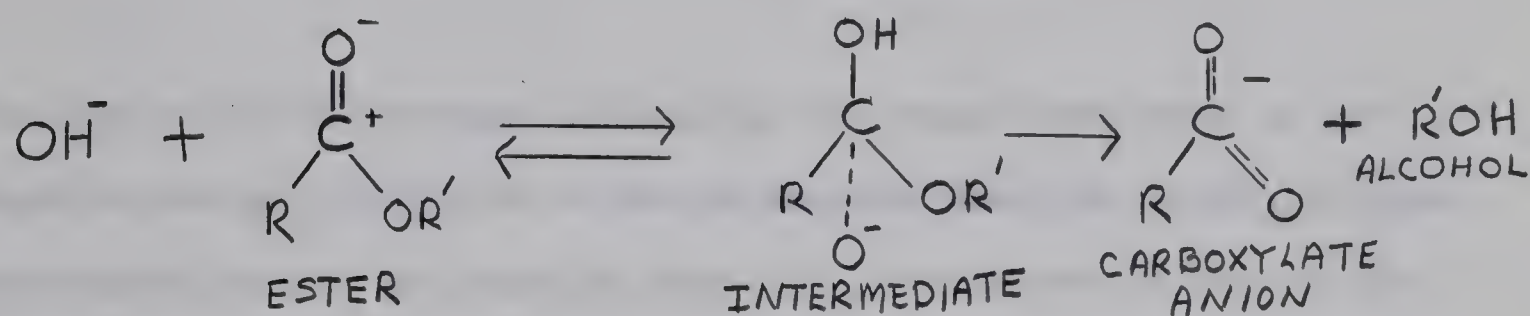
(2) Inhibition by protohemin should be competitive in nature. (3) The inhibitory effect of protohemin should be confined to the transcription process. In Chapter IV experiments have been designed to investigate the above consequences of the hypothesis.

CHAPTER II RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND PORPHYRIA-INDUCING ACTIVITY

A. Aliphatic Compounds

The objective of the experiments described in this part of the thesis was to test the hypothesis that the critical feature for porphyria-inducing activity in the AIA (Fig. 5B) series of compounds was an ester or amide group sterically hindered from hydrolysis by acid or base.

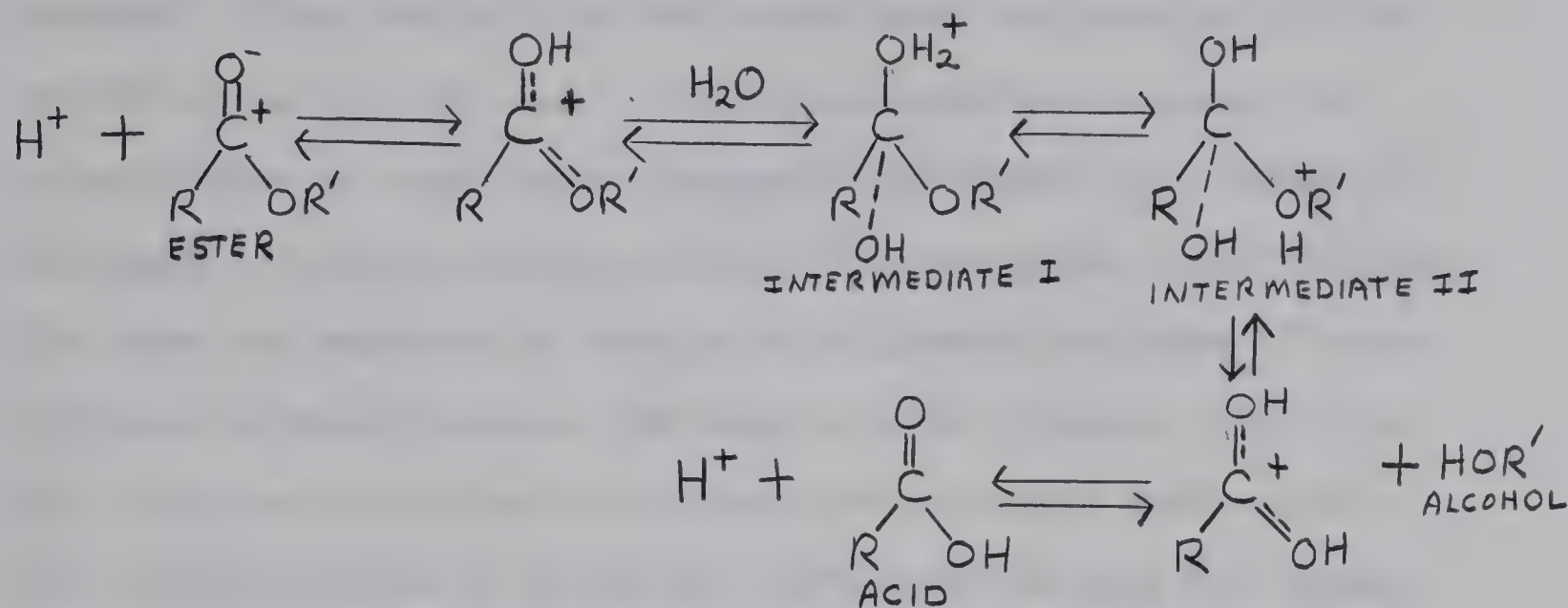
The alkaline hydrolysis of esters is considered to proceed by the following mechanism (Morrison & Boyd, 1966; Newman, 1956).



The nucleophilic hydroxyl ion attacks the carbonyl carbon which is the positive end of the dipole in the unsaturated function. In the ester the groups R and OR' lie in the same plane as the carbon-oxygen double bond and the bond angles are 120°. The hydroxyl ion therefore attacks the carbonyl carbon from either above (or below) the plane of the paper and the substituents (R, OR', O⁻) move below (or above) the plane of the paper until the tetrahedral intermediate is obtained. Elimination of a molecule of alcohol from the tetrahedral intermediate completes the hydrolysis. The slow step in the reaction sequence is the addition of hydroxyl ion to the carbonyl carbon to form the tetrahedral intermediate.

The acidic hydrolysis of esters is considered to proceed by

the following mechanism (Morrison & Boyd, 1966):

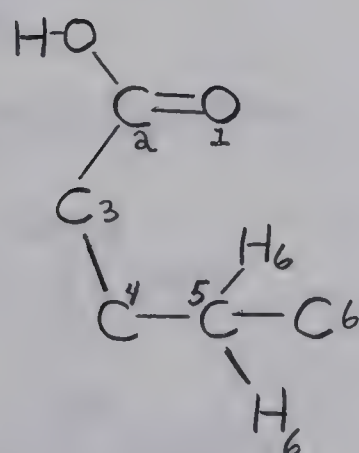


The electrophilic hydrogen ion attacks the oxygen atom which is the negative end of the dipole in the unsaturated function forming a planar protonated ester. Nucleophilic attack by water above (or below) the plane of the paper results in formation of the bulky tetrahedral intermediate I which is in equilibrium with intermediate II. Release of an alcohol molecule from intermediate II results in the formation of the planar protonated acid which releases a proton. As in the case of alkaline hydrolysis the slow step is the formation of the bulky tetrahedral intermediate I.

In order to estimate the degree of steric hindrance to hydrolysis in the compounds selected for study we have utilized several concepts formulated by Newman (1956). Newman has pointed out that steric factors in a given compound are believed to be the same for esterification and hydrolysis under acidic and basic conditions. It follows that an assessment of steric hindrance to hydrolysis in a molecule is possible by comparing the rate of esterification of the

parent acid with the rate of esterification of acetic acid (taken as a standard). Thus, the ratio of the second order rate constant (k) for esterification of acetic acid to the second order rate constant for esterification of a particular carboxylic acid (RCOOH) is a measure of the degree of steric hindrance to esterification in the latter molecule. The larger the magnitude of this ratio the greater the degree of steric hindrance to esterification. The data in table I (Newman, 1956) show that the value of this ratio is highest for the highly branched acid, 10, thus showing that it is the most highly hindered acid with respect to esterification. Conversely acid, 1, is the least hindered with respect to esterification.

Newman (1956) has also formulated the empirical rule of six to estimate the degree of steric hindrance to esterification. Newman numbered the carbon atoms in a saturated carboxylic acid consecutively starting with the carbonyl oxygen as one (Fig. 9A). The number of atoms in the six position is the six-number.



(A)



(B)

Fig. 9

TABLE I. DATA ON ACID-CATALYZED ESTERIFICATION OF ALIPHATIC ACIDS

Acid	$\frac{k^{40^\circ} \text{CH}_3\text{COOH}}{k^{40^\circ} \text{RCOOH}}$	Atoms in Position 6		
		C	H	Total
1. CH_3COOH	1	0	0	0
2. $\begin{array}{c} \text{CH}_3 \\ \diagdown \\ \text{C} \\ \diagup \\ \text{CH}_3 \end{array} \text{HCOOH}$	10.1	0	3	3
3. $\begin{array}{c} \text{CH}_3 \\ \diagdown \\ \text{CH} \\ \diagup \\ \text{CH}_3 \end{array} \text{CH}_2\text{COOH}$	8.57	0	6	6
4. $(\text{CH}_3)_3\text{CCOOH}$	26.8	0	0	0
5. $\begin{array}{c} \text{H}_5\text{C}_2 \\ \diagdown \\ \text{H} \\ \diagup \\ \text{H}_5\text{C}_2 \end{array} \text{CCOOH}$	100	0	6	6
6. $\begin{array}{c} n\text{-C}_3\text{H}_7 \\ \diagdown \\ \text{H} \\ \diagup \\ n\text{-C}_3\text{H}_7 \end{array} \text{CCOOH}$	108	2	4	6
7. $(\text{CH}_3)_3\text{CCH}_2\text{COOH}$	42.7	0	9	9
8. $\begin{array}{c} \text{H}_3\text{C} \quad \text{H}_5\text{C}_2 \\ \diagdown \quad \diagup \\ \text{C} - \text{C} \\ \diagup \quad \diagdown \\ \text{H}_3\text{C} \quad \text{H} \end{array} \text{COOH}$	1692	0	9	9
9. $(\text{CH}_3)_3\text{C}-\begin{array}{c} \text{CH}_3 \\ \\ \text{C} \\ \\ \text{CH}_3 \end{array} \text{COOH}$	7765	0	9	9
10. $\begin{array}{c} \text{H}_3\text{C} \\ \diagdown \\ \text{CH} \\ \diagup \\ \text{H}_3\text{C} \end{array} \text{H}-\begin{array}{c} \text{H} \\ \\ \text{C} \\ \\ \text{H}_3\text{C} \end{array} \text{CH}_2\text{COOH}$	too slow to measure	0	12	12

The rule of six states: "In reactions involving addition to an unsaturated function containing a double bond, the greater the number of atoms in the six position the greater will be the steric effect." In table I is shown the six-number of all the acids considered. There is a rough correspondence between the degree of steric hindrance determined by the six-number and that determined by the magnitude of the ratio $k^{40^\circ} \text{CH}_3\text{COOH} / k^{40^\circ} \text{RCOOH}$. Thus where this ratio for a particular acid is unknown the six-number may be used as an approximate index of steric hindrance. However, it is quite clear that where data is available the ratio $K^{40^\circ} \text{CH}_3\text{COOH} / k^{40^\circ} \text{RCOOH}$ is the more accurate index since the six-number is not always an accurate reflection of the degree of steric hindrance in a compound. The reason why atoms in the six position are effective in providing steric hindrance can be understood from the following considerations: examination of the conformation presented in (Fig. 9B) which is a Courtald model of the compound shown in (Fig. 9A) shows that the carbon atom labelled, 6, and the hydrogen atoms labelled, 6, closely approach the carbonyl carbon labelled, 2. They thus shield the carbonyl carbon from attack by nucleophiles on one side of the unsaturated function. If the other side of the unsaturated function is also shielded in a similar manner then hydrolysis of the ester is extremely difficult since no nucleophilic attack can take place at the carbonyl carbon. This follows from consideration of the mechanism of acid or base catalyzed ester hydrolysis. In some molecules only one side of the unsaturated function may be shielded from attack. In such a case attack may proceed from the other side in a normal manner. However, the presence of the six-substituents now hinders the formation

of the required tetrahedral intermediate and hydrolysis is thus rendered more difficult. By increasing the branching on the α , β , and γ carbon atoms of an ester or amide, the six-number is increased and there is greater steric hindrance to hydrolysis. The rule of six is applicable for estimating the degree of steric hindrance to esterification in unsaturated acids and in amides. Accordingly we have estimated the degree of steric hindrance to hydrolysis by the magnitude of the six-number and by the ratio of the rate of esterification of acetic acid to the parent acid when this data was available.

AIA (Fig. 5B), a potent porphyria-inducing drug, has a six-number of eight suggesting a considerable degree of steric hindrance to hydrolysis of the amide group. If the hypothesis of Hirsch et al. (1967) is correct then a reduction in the steric hindrance to hydrolysis in compounds of this type should lead to a reduction in the ability to induce porphyria. For this reason the following compounds were selected (table II) and tested for porphyria-inducing activity: allylisopropylacetamide (six-number = 8), di-n-propylacetamide (six-number = 6), α -ethylbutyramide (six-number = 6), α -methylbutyramide (six-number = 3), and β -methylbutyramide (six-number = 6). On the basis of the ratio $k^{40^\circ} \text{CH}_3\text{COOH} / k^{40^\circ} \text{RCOOH}$ and the six-number it was anticipated that the compounds could be arranged in the following order (table II) with respect to decreasing magnitude of steric hindrance to hydrolysis. Accordingly, if the hypothesis of Hirsch et al. (1967) is correct the same order should be observed with respect to porphyria-inducing activity.

According to Hirsch et al. (1967) the replacement of an amide

TABLE II. ALIPHATIC AMIDES ARRANGED IN ORDER OF DECREASING STERIC
HINDRANCE TO HYDROLYSIS

Compound	$\frac{k^{40^\circ} \text{CH}_3\text{COOH}}{k^{40^\circ} \text{RCOOH}}$	Six-Number
Allylisopropylacetamide	†	8
Di-n-propylacetamide	108	6
α-Ethylbutyramide	100	6
α-Methylbutyramide	10.1	3
β-Methylbutyramide	8.57	6

†No data available

group by an ester group in AIA (Fig. 5B) and related analogues should result in no change in porphyria-inducing activity. For this reason ethyl allylisopropyl acetate (Fig. 10A) and ethyl di-n-propyl acetate (Fig. 10B) were tested for porphyria-inducing activity. Since ethyl allylisopropyl acetate has the same degree of steric hindrance to hydrolysis as the corresponding amide (AIA) it was anticipated that it would have the same porphyria-inducing activity. Similarly ethyl di-n-propyl acetate was expected to have the same porphyria-inducing activity as di-n-propylacetamide.

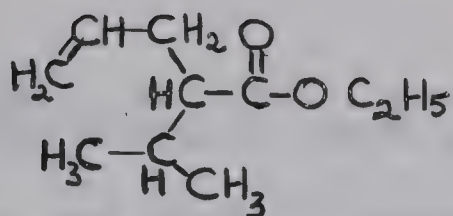
Methyl di-t-butyl acetate (Fig. 10C) and methyl isopropylisobutyl acetate (Fig. 10D) are two esters severely hindered with respect to hydrolysis. They should therefore be highly active and were thus tested for porphyria-inducing activity.

Granick (1966) measured the activity of a series of aliphatic di-esters and found that diethyl β -methylglutarate (Fig. 10E) was active while diethylglutarate (Fig. 10F) was inactive. We measured and compared the activity of diethyl β,β -dimethylglutarate (Fig. 10G) and diethyl α,α' -dimethylglutarate (Fig. 10H) to the activity of dimethyl β -methylglutarate and diethylglutarate. Diethyl β,β -dimethylglutarate (six-number = 9) should have considerable activity whereas diethyl α,α' -dimethylglutarate (six-number = 3) should be inactive.

Experimental

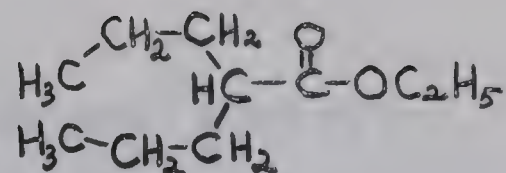
i. Source of Compounds

Allylisopropylacetamide was obtained from Hoffman laRoche, Montreal. Di-n-propylacetamide, α -ethylbutyramide, α -methylbutyramide,



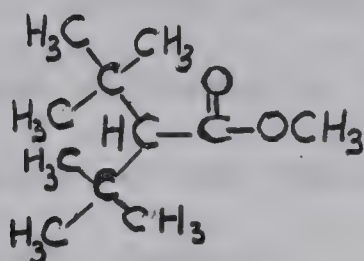
(A)

Ethyl Allylisopropylacetate



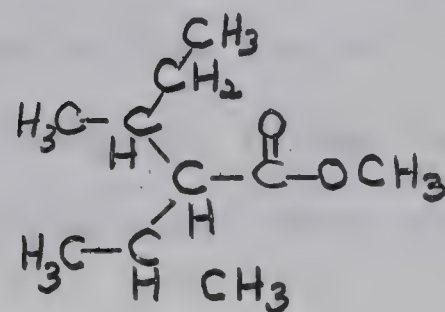
(B)

Ethyl di-n-propylacetate



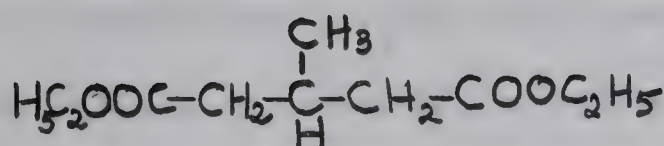
(C)

Methyl di-t-butylacetate

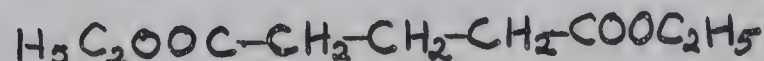


(D)

Methyl isopropylisobutylacetate

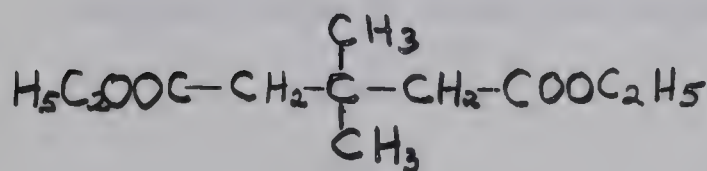


(E)

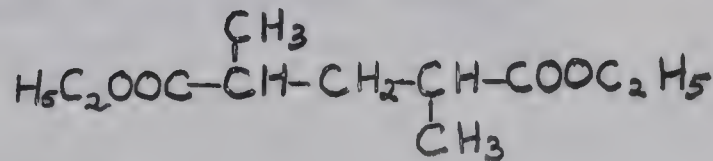
Diethyl β -methylglutarate

(F)

Diethyl glutarate



(G)

Diethyl β,β -dimethylglutarate

(H)

Diethyl α,α' -dimethylglutarate

Fig. 10 Aliphatic Mono- and Diesters Tested for
Porphyrin-Inducing Activity

diethyl β -methylglutarate, ethyl allylisopropylacetate, ethyl di-n-propylacetate, and diethyl β',β -dimethylglutarate were synthesized by Mr. G. L. Bubbar in our laboratory. β -methylbutyramide was donated by Professor C. Rimington, University College, London. Methyl di-t-butylacetate and methyl isopropylisobutylacetate were obtained from Dr. C. K. Hancock, Department of Chemistry, Texas A & M University, College Station, Texas. Diethylglutarate and diethyl α,α' -dimethylglutarate were purchased from Aldrich Chemical Company.

ii. Testing of Compounds for Porphyria-Inducing Activity in Cultures of Chick Embryo Liver Cells

(All procedures in this technique were carried out using standard microbiological aseptic techniques.)

Preparation of Reagents

a. Calcium and magnesium-free Earle's medium:

This medium contained the following substances dissolved in one litre of water and adjusted to pH 6.8 for storage:

NaCl	6.8 g
KCl	0.4 g
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	0.125 g
Dextrose	1.0 g
NaHCO_3	2.2 g

This solution was sterilized through a Millipore filter (porosity 0.45 μ).

b. Pangestin Solution

One gram of Pangestin (1:75 Difco certified), which is an active preparation of the enzymes of the pancreas (principally amylase, trypsin, and steapsin) was added to calcium and magnesium-free Earle's medium (100 ml) and allowed to stand for twelve hours. pH was adjusted to 6.8 and the suspension filtered by gravity. The solution was finally filtered through a Millipore filter. The filtrate was divided into 5 ml aliquots which were stored in screw cap culture tubes at -15° .

c. Medium for Culturing Liver Cells

The following compounds were added to a 150 ml Pyrex screw cap bottle:

1. 100 ml Eagle's Basal medium without phenol red (Microbiological Associates).
2. 1 ml Glutamine solution (Microbiological Associates).
This solution was kept at -15° until used.
3. 10,000 I.U. buffered penicillin G (crystalline potassium salt, Eli Lilly and Co.) dissolved in 0.25 ml sterile water.
4. 10 mg streptomycin sulfate (U.S.P., Eli Lilly and Co.) dissolved in 0.25 ml sterile water.
5. 2000 U. mycostatin (Squibb) suspended in 0.25 ml sterile water.
6. 10 ml Fetal Bovine Serum (Microbiological Associates).

d. Preparation of Enzyme Solution for Digestion of Cells

Calcium and magnesium-free Earle's medium (6 ml) was added to a vial containing sterile, crystallized, and lyophilized trypsin (100 mg, Worthington Biochemical). Three ml of Pangestin solution were added to the vial and the contents of the vial mixed whereupon a clear solution resulted. This enzyme mixture was always used immediately after preparation.

e. Preparation of Liver Cells

The livers from seventeen day old chick embryos were washed three times with calcium and magnesium-free Earle's medium in a small petri dish. The livers were transferred to a second petri dish containing the enzyme mixture and cut up into small pieces with a razor blade. After incubating the mixture on a serological bath at 37° until most of the cells were separated (15-30 min), the suspension was transferred to a conical centrifuge tube whereupon the larger particles settled to the bottom of the tube (5 minutes). The suspension was then transferred to a second conical centrifuge tube. Aliquots (0.05 ml) of the supernatant suspension of free cells were inoculated into aluminum capped culture vials (O.D. 19 x 63 mm) containing a glass cover slip (size 5/8 inch, thinness 0) and 1 ml of culture medium. The cells were incubated at 37° in an atmosphere of moist 95% air : 5% carbon dioxide for twenty-four hours.

f. Counting of the Liver Cells

Immediately after the addition of the liver cells to the aluminum capped culture vials a small aliquot of the liver cell suspension was taken, diluted 1:100 with 0.9% saline in a serological pipette, and counted on a hemocytometer. The average number of cells found was 3.4×10^5 per 0.05 ml of cell suspension.

g. Addition of Chemicals to Liver Cells

The culture medium was removed from each culture vial by means of a Pasteur pipette and replaced with fresh medium (1 ml). The chemicals under investigation dissolved in 95% ethanol (1-5 μ) were added to the liver cells by means of lambda pipettes. After the addition of the chemicals the culture vials were returned to the incubator for approximately twenty-four hours.

h. Measurement of Fluorescence Intensity

The cover slips were removed from the culture vials, inverted onto microscope slides, and gently blotted with filter paper. The cover slips were sealed to the slides with molten paraffin, washed with distilled water, and examined for the presence of porphyrins with a fluorescence microscope.

i. Results Obtained on Testing Compounds for Porphyrin-Inducing Activity

In each experiment a drug was tested in three separate vials at a particular concentration. Each drug was tested in the above manner in two or three separate experiments. To

eliminate bias in scoring of the fluorescence intensity, the experiment was arranged so that the observer did not know which drug was added to a particular vial. Moreover, in each experiment a large number of vials were included to which no drug had been added. The fluorescence intensity was scored as follows: +4, all colonies fluoresce intensely; +3, most colonies fluoresce intensely; +2, most colonies fluoresce partially; +1, some colonies fluoresce partially. Fig. 11A is a photograph of the cells taken by means of a phase contrast microscope at a magnification of 400 diameters and shows a small portion of a monolayer colony. The nucleus and nucleolus are clearly differentiated from the surrounding cytoplasm. The cytoplasm of the peripheral cells shows fingerlike extensions spreading over the surface of the glass. Such a picture is suggestive of healthy cellular growth. In Fig. 11B is shown a photograph of a colony of the cells taken by means of a fluorescence microscope twenty-four hours after the addition of a porphyria-inducing drug.

In table III the individual values of the fluorescence intensity obtained for each drug are recorded.

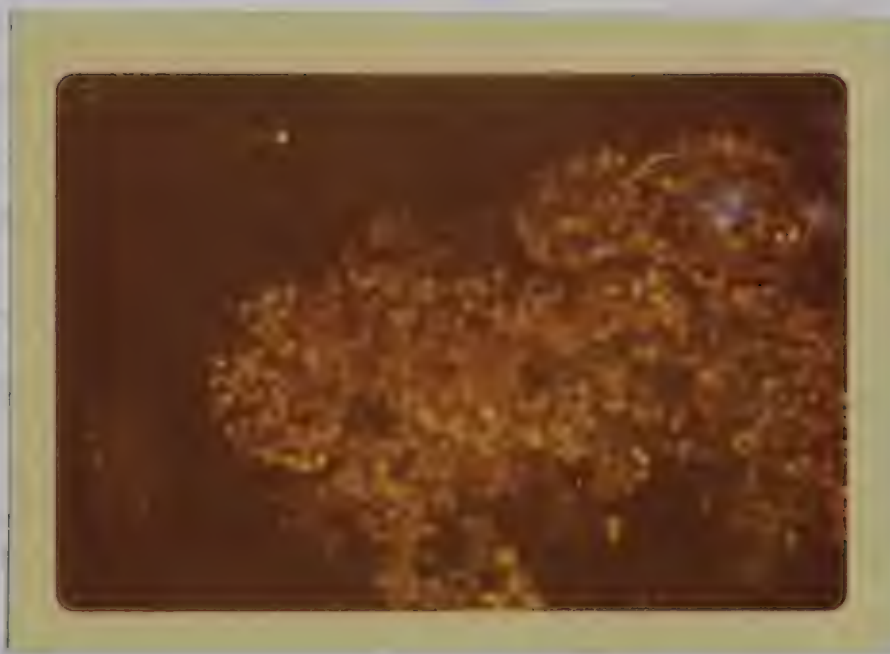
In table IV the average value of the fluorescence intensity for each compound is shown and in addition the six-number and the ratio $k^{40^\circ} \text{CH}_3\text{COOH} / k^{40^\circ} \text{RCOOH}$ are recorded.

Discussion

The results shown in table IV reveal that in the case of



(A)



(B)

Fig. 11

Photographs Taken under Phase Contrast (A) and Fluorescence Microscopy (B) of Chick Embryo Liver Cells Growing in Primary Culture.

TABLE III. PORPHYRIN ACCUMULATION IN PRIMARY CULTURE OF CHICK EMBRYO
LIVER CELLS INDUCED BY A VARIETY OF CHEMICALS AND MEASURED
BY FLUORESCENCE MICROSCOPY

Compound Tested	Concentration ($\mu\text{g/ml}$)	Fluorescence Intensity*		
		Expt. 1	Expt. 2	Expt. 3
Allylisopropylacetamide	100	3,3,4	2,2.5,3	3.5,3,3.5
	20	2,2,2.5		2,2.5,2
	5	0.5,1	1,1	
	1		tr,0.5	tr,tr
Di-n-propylacetamide	100	2,2,3	3,2,3	2,2.5,3
	50	2,2,3	3,2,2	2,2,2
	25	2	2	1.5,1.5, 1.5
α -Ethylbutyramide	100	3.5,2.5, 2.5	1,1.5, 1.5	3.5,2.5, 2
	10	0,tr,0	tr(2),0	0,tr,0
α -Methylbutyramide	100	0,tr(2)	0,tr,0	0,0,0
	10	0,tr,0	0,0,0	0,0,0
β -Methylbutyramide	100	0,0,0	0,0,0	
	50	0,0,0	tr,0,0	
	25	0		
Ethyl allylisopropylacetate	100	0,0,0	0,tr,0.5	0.5,0.5, 0.5
	20	0,0,0	tr,0,0	0,0,0
Ethyl di-n-propylacetate	100	0,0,0	0,0,0	
	50	0,0,0	0,0,tr	
	25	0	0	
Methyl di-t-butylacetate	100	1,1,0.5	tr,0,0	0.5, tr(2)
	20	tr(2),0	0,tr(2)	tr,0,0
Methyl isopropylisobutyl- acetate	100	0.5,0,0	0,tr(2)	0.5, tr(2)
	20	0,0,0	tr(2),0	0,0,0

TABLE III (continued). PORPHYRIN ACCUMULATION IN PRIMARY CULTURE OF CHICK EMBRYO LIVER CELLS INDUCED BY A VARIETY OF CHEMICALS AND MEASURED BY FLUORESCENCE MICROSCOPY

Compound Tested	Concentration ($\mu\text{g/ml}$)	Fluorescence Intensity*		
		Expt. 1	Expt. 2	Expt. 3
Diethyl β,β -dimethyl- glutarate	200	2.5,2,2.5	1.5,2,3	4,4,3
	40	2,2,0.5	0.5,0.5, tr	1.5,1.5, 1.5
Diethyl β -methylglutarate	200	2,0,tr	tr(3)	1,0.5, 1.5
	40	0,0,0	0,0	0,0,0
Diethyl α,α' -dimethyl- glutarate	200	tr(3)	0,0,tr	
	40	0,0	0,0,0	
Diethyl glutarate	200	tr,0,0	0,0,0	
	40	0,0,0	0,0,0	

* tr = trace

TABLE IV. PORPHYRIN ACCUMULATION IN PRIMARY CULTURE OF CHICK EMBRYO LIVER
CELLS INDUCED BY A VARIETY OF CHEMICALS AND MEASURED BY
FLUORESCENCE MICROSCOPY

Compound Tested	Concentration ($\mu\text{g/ml}$) ($\text{M} \times 10^{-5}$)		$\frac{k^{40^\circ} \text{CH}_3\text{COOH}}{k^{40^\circ} \text{RCOOH}}$	Six- Num- ber	Intensity of Fluor- escence (mean)
Allylisopropylacetamide	100	71	†	8	3
	20	14			2
	5	4			1
	1	0.7			tr
Di-n-propylacetamide	100	70	108	6	2.5
	50	35			2
	25	18			2
α -Ethylbutyramide	100	87	100	6	2
	10	9			0
α -Methylbutyramide	100	99	10.1	3	0
	10	10			0
β -Methylbutyramide	100	99	8.57	6	0
	50	50			0
	25	25			0
Ethyl allylisopropyl- acetate	100	59	†	8	tr
	20	12			0
Ethyl di-n-propylacetate	100	58	108	6	0
	25	15			0
Methyl di-t-butylacetate	100	54	too slow to measure	18	0.5
	20	11			0
Methyl isopropylisobutyl- acetate	100	59	too slow to measure	12	tr
	20	12			0

TABLE IV (continued). PORPHYRIN ACCUMULATION IN PRIMARY CULTURE OF CHICK EMBRYO LIVER CELLS INDUCED BY A VARIETY OF CHEMICALS AND MEASURED BY FLUORESCENCE MICROSCOPY

Compound Tested	Concentration		$\frac{k^{40^\circ} \text{CH}_3\text{COOH}}{k^{40^\circ} \text{RCOOH}}$	Six- Num- ber	Intensity of Fluor- escence (mean)
	($\mu\text{g/ml}$)	($\text{M} \times 10^{-5}$)			
Diethyl β,β -dimethyl- glutarate	200	93	†	9	3
	40	19			1
Diethyl β -methylglutarate	200	99	†	6	1
	40	19			0
Diethyl α,α' -dimethyl- glutarate	200	93	†	3	tr
	40	19			0
Diethyl glutarate	200	106	†	3	0
	40	21			0

† Data not available

aliphatic mono-amides the porphyria-inducing activity is related to the degree of steric hindrance in the molecule as estimated by the ratio $k^{40^\circ} \text{CH}_3\text{COOH} / k^{40^\circ} \text{RCOOH}$. The activity is also related to the magnitude of the six-number with the exception of β -methylbutyramide (six-number = 6) which appears to be anomalous. However as discussed previously the six-number is only an approximation of the degree of steric hindrance whereas the ratio $k^{40^\circ} \text{CH}_3\text{COOH} / k^{40^\circ} \text{RCOOH}$ provides a more accurate assessment of this parameter. The results obtained are thus in accordance with the hypothesis of Hirsch et al. (1967).

The aliphatic mono-ester, ethyl di-n-propyl-acetate (six-number = 6) was found to be inactive while ethyl allylisopropylacetate (six-number = 8) showed only slight activity. These results were unexpected since the corresponding amides, di-n-propylacetamide (six-number = 6) and allylisopropylacetamide (six-number = 8) were active. Moreover the highly hindered esters methyl isopropylisobutylacetate (six-number = 12) and methyl di-t-butylacetate (six-number = 18) showed only slight activity. The results obtained with the aliphatic di-esters are in accordance with the hypothesis of Hirsch et al. (1967). Thus diethyl β, β -dimethylglutarate (six-number = 9) showed considerable activity whereas diethyl α, α' -dimethylglutarate (six-number = 3) was inactive. In comparing the results obtained with the aliphatic mono- and di-esters it is apparent that one sterically hindered ester group confers slight activity on a molecule, while a second sterically hindered ester group in a molecule reinforces this activity. Although the hypothesis of Hirsch et al. (1967) would appear to explain the activity of the series of aliphatic mono-amides and di-esters it does not explain the inactivity of the aliphatic mono-esters indicating that

factors other than steric requirements may be necessary for activity in this group of compounds. It is clear by examination of the results in table IV that a similarly hindered amide and ester group are not equivalent with respect to porphyria-inducing activity in this series of compounds.

B. Aromatic Compounds

Introduction

In a previous study (Hirsch et al., 1967) the high activity of diethyl 2,3,5,6-tetramethylterephthalate (Fig. 12A) was attributed to the fact that the molecule contained two ethoxycarbonyl substituents, each of which was sterically hindered from hydrolysis by two ortho-methyl substituents. In the same study the activity of ethyl 2,4,6-trimethylbenzoate (Fig. 12B) was attributed to the ethoxycarbonyl substituent sterically hindered from hydrolysis by two ortho-methyl substituents. In addition, the replacement of the ester group in ethyl 2,4,6-trimethylbenzoate by an amide group (Fig. 12C) resulted in retention of porphyria-inducing activity suggesting that in this group of compounds the critical feature for porphyria-inducing activity is an ester or amide group which is sterically hindered from hydrolysis.

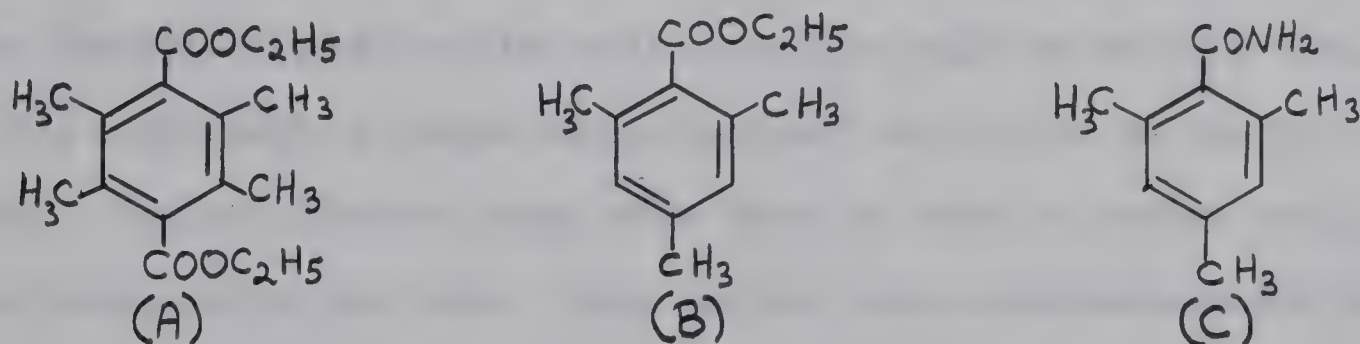


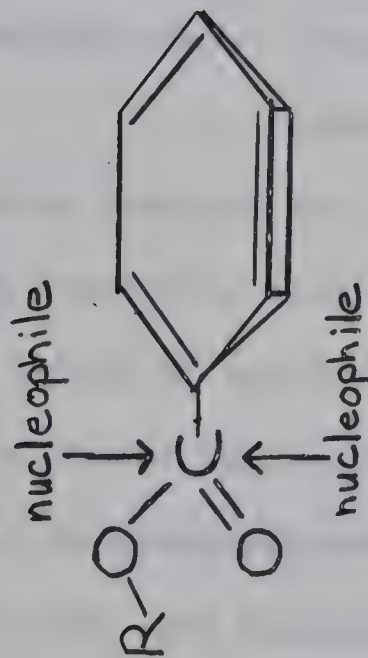
Fig. 12

The objective of the experiments conducted in this part of the thesis was

to test this hypothesis. This was done by selecting compounds with varying degrees of steric hindrance to the hydrolysis of ester or amide groups and measuring their porphyria-inducing activity.

Ethyl 2,4,6-trimethylbenzoate (Fig. 12B) has played an important historical role in the development of concepts of steric hindrance. It has been known since the end of the last century that the hydrolysis of ethyl benzoate is blocked by the presence of substituents in the two ortho positions. When only a single ortho substituent is present, the ester is readily hydrolyzed but at a slower rate than the corresponding para isomer. Increasing the bulk of the ortho substituent(s) results in further hindrance to hydrolysis (Newman, 1956).

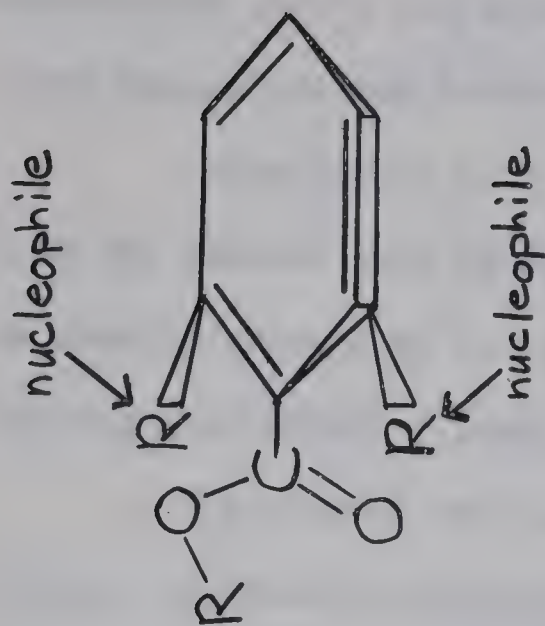
Kadesch (1944) pointed out that where hydrolysis of an aromatic ester occurs the attacking reagent must approach the carbonyl carbon from a direction perpendicular to the plane defined by the unsaturated function. As long as this plane is essentially coplanar with the ring there is little steric hindrance from the ortho groups since the reagent has an unhindered avenue of approach perpendicular to the benzene ring (Fig. 13A). However, if the ortho groups are large enough to prevent free rotation about the bond connecting the unsaturated function to the ring then the plane containing this function must lie at an angle to the ring (Fig. 13B). In this orientation a perpendicular approach is hindered by the ortho groups. One sufficiently large ortho group is able to prevent co-planarity of a function with the ring. Reaction with such a monosubstituted derivative is still possible by a perpendicular approach from the side opposite to the ortho group. This explanation is consistent with the fact that in aromatic esters unsubstituted esters are readily hydrolyzed, monoortho-



(A)

No Bulky Ortho Substituents:

The ester function lies in the same plane as the benzene ring. The nucleophile can readily attack the carbonyl carbon from a direction perpendicular to the plane of the benzene ring.



(B)

Two Bulky Ortho Substituents:

The ester function lies in a plane perpendicular to the benzene ring. The nucleophile must approach the carbonyl carbon from a direction perpendicular to the plane of the ester group. Access is blocked by the bulky ortho substituents (R).

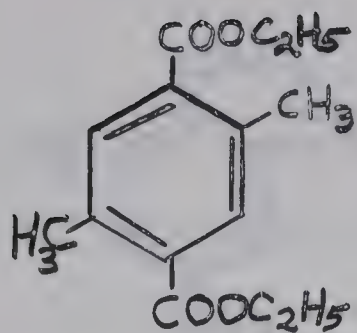
Fig. 13 Steric Hindrance in Aromatic Esters

substituted esters are hydrolyzed with more difficulty and diorthosubstituted esters are not hydrolyzed under normal acidic or basic conditions.

When one or more carbon atoms separate the carbonyl carbon from the benzene ring in diorthosubstituted esters the resulting ester is readily hydrolyzed since the carbonyl carbon is readily available for nucleophilic attack (Fieser & Fieser, 1956).

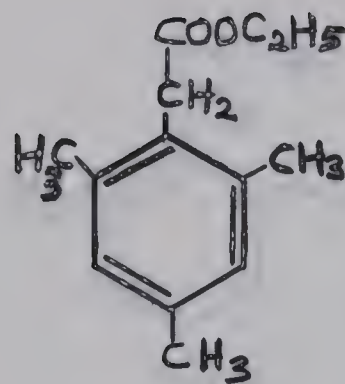
In view of the above considerations it was anticipated that diethyl 2,5-dimethylterephthalate (Fig. 14A) in which the steric hindrance to hydrolysis of the ethoxycarbonyl substituents is largely removed would be considerably less active than diethyl 2,3,5,6-tetramethylterephthalate (Fig. 12A). Moreover, it was anticipated that ethyl 2-(2,4,6-trimethylphenyl)ethanoate (Fig. 14B), ethyl 3-(2,4,6-trimethylphenyl)propanoate (Fig. 14C), ethyl 3,5-dimethylbenzoate (Fig. 14D), and ethyl benzoate (Fig. 14E) would be considerably less active than ethyl 2,4,6-trimethylbenzoate (Fig. 12B).

2,4,6-Trimethylbenzamide (Fig. 15A) is active as a porphyria-inducing compound and this activity was ascribed to the amide group being sterically hindered from hydrolysis (Hirsch et al., 1967). Therefore relief of steric hindrance to hydrolysis in this type of compound should result in inactivity. It was anticipated, therefore, that 2-(2,4,6-trimethylphenyl)propanamide (Fig. 15C), 3,5-dimethylbenzamide (Fig. 15D), and benzamide (Fig. 15E) would be considerably less active than 2,4,6-trimethylbenzamide (Fig. 15A). Results of these experiments are recorded in table V.



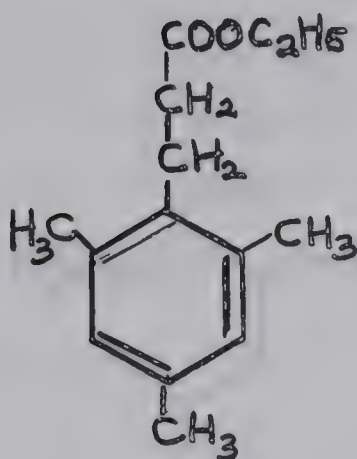
(A)

Diethyl 2,5-dimethyl-
terephthalate



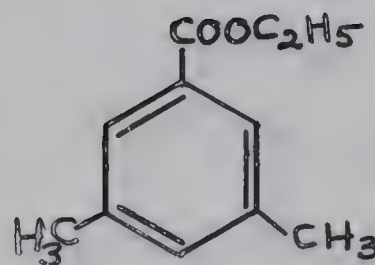
(B)

Ethyl 2-(2,4,6-trimethylphenyl)-
ethanoate



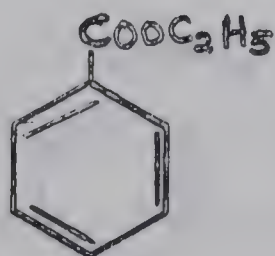
(C)

Ethyl 3-(trimethylphenyl)-
propanoate



(D)

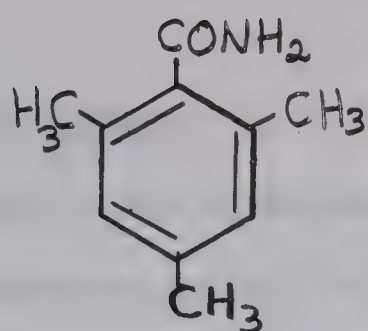
Ethyl 3,5-dimethylbenzoate



(E)

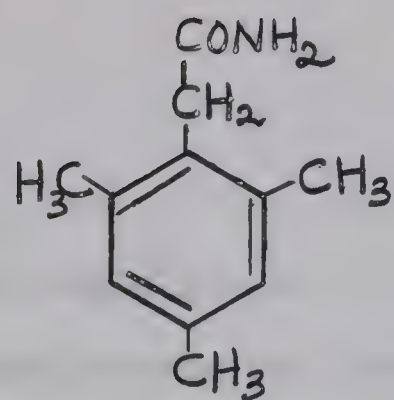
Ethyl benzoate

Fig. 14 Aromatic Esters with Varying Degrees of
Steric Hindrance to Hydrolysis of the
Ester Function(s)



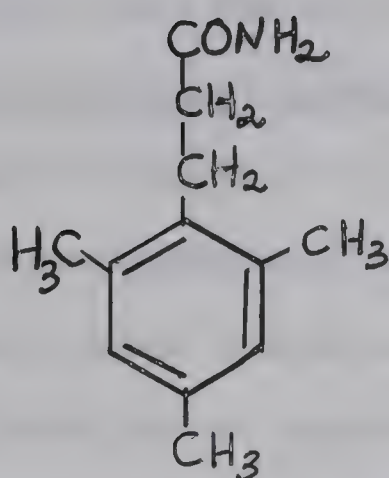
(A)

2,4,6-Trimethylbenzamide



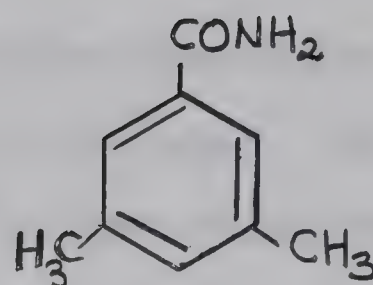
(B)

2-(2,4,6-Trimethylphenyl)-ethanamide



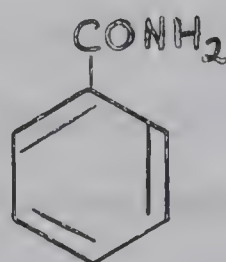
(C)

3-(2,4,6-Trimethylphenyl)-propanamide



(D)

3,5-Dimethylbenzamide



(E)

Benzamide

Fig. 15 Aromatic Amides with Varying Degrees of Steric Hindrance to Hydrolysis of the Amide Function

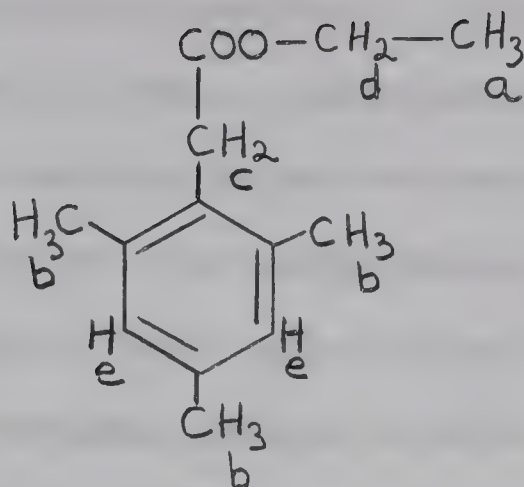
Experimental

i. Source of Compounds

Diethyl 2,3,5,6-tetramethylterephthalate, ethyl 2,4,6-trimethylbenzoate, ethyl 3-(2,4,6-trimethylphenyl)propanoate, ethyl 3,5-dimethylbenzoate, 2,4,6-trimethylbenzamide, 3-(2,4,6-trimethylphenyl)propanamide, and 3,5-dimethylbenzamide were synthesized by Mr. G. L. Bubbar in our laboratory. Details of the properties of these compounds have been described (Schneck et al., 1968).

Ethyl 2-(2,4,6-trimethylphenyl)ethanoate

Thionyl chloride (2 ml, 0.028 moles) was added dropwise to 2-(2,4,6-trimethylphenyl)ethanoic acid (2.0 g, 0.012 moles). The mixture was allowed to reflux three hours and was then transferred to a bulb distillation apparatus with ether. After removal of the ether the acid chloride distilled as a colorless oil (0.0088 moles, 77%), b.p. 152-160°/14 mm. The acid chloride (0.9 g, 0.0088 moles) was added to absolute ethanol (20 ml, 0.34 moles) and refluxed for three hours. Excess ethanol was removed by distillation and ether (100 ml) added. The ether solution was washed with 5% NaHCO₃ and water and dried (Na₂SO₄). The ether solution was transferred to a bulb distillation apparatus and the ether removed. Distillation afforded the product as a colorless liquid (0.78 g, 82%), b.p. 135-138°/13 mm. Infrared (liquid film): Max. 1725 cm⁻¹ (ester C=O stretching). The nuclear magnetic resonance spectral data are given below and observations regarding the spectrum are shown. The spectrum was determined in CCl₄: external reference--tetramethylsilane; oscillator frequency 60 Mc.

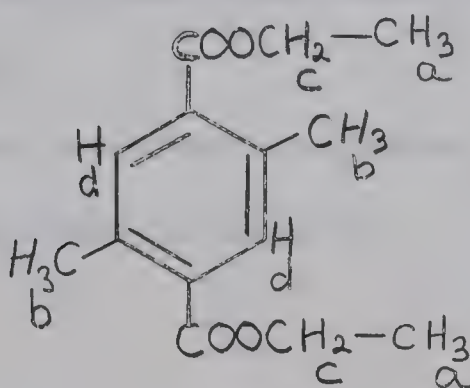


<u>τ value</u>	<u>Proton Assignment</u>	<u>Relative Peak Area</u>	
		<u>Found</u>	<u>Theoretical</u>
8.82	a	2.8	3
7.79	b	8.8	9
6.52	c	2	2
6.01	d	2	2
3.32	e	1.8	2

Diethyl 2,5-dimethylterephthalate

Magnesium dried ethanol (300 ml, 5.2 moles) was added to 2,5-dimethylterephthalic acid (5 g, 0.026 moles) and HCl gas was

passed into the mixture. After refluxing for two hours the mixture was cooled whereupon crystals separated. The crystals were dissolved in ether and washed with 5% NaHCO_3 , water and then dried (MgSO_4). After removal of the ether the diester was twice crystallized from hot ethanol and appeared as white needle-shaped crystals, m.p. $84-87^\circ$. Infrared (carbon tetrachloride): max. 1725 cm^{-1} (ester $\text{C}=\text{O}$ stretching). The nuclear magnetic resonance spectrum is given below and observations regarding the spectrum shown. The spectrum was determined in CCl_4 : external reference--tetramethylsilane; oscillator frequency 60 Mc.



<u>τ value</u>	<u>Proton Assignment</u>	<u>Relative Peak Area</u>	
		<u>Found</u>	<u>Theoretical</u>
8.60	a	3.15	3
7.48	b	2.95	3
5.74	c	2.05	2
2.42	d	1.00	1

2-(2,4,6-trimethylphenyl)ethanamide

2,4,6-Trimethylbenzoyl chloride (1.3 g, 0.0066 moles) was dissolved in ether (50 ml) and NH_3 gas was bubbled into the solution for two hours whereupon the amide separated as white flakes. After three crystallizations from ethanol-water the amide appeared as white needles, m.p. $211.5\text{--}213.5^\circ$ (decomp.). Buchner and Schattenhammer (1920) record a m.p. of 210° . Infrared (chloroform): max. 1670 cm^{-1} (amide I), 1570 cm^{-1} (amide II), 3550 and 3420 cm^{-1} (N-H stretching).

Anal. Calcd. for $\text{C}_{11}\text{H}_{15}\text{NO}$: N, 7.90 Found: N, 7.53 (Vogel, 1961).

ii. Testing of Compounds for Porphyrin-Inducing Activity in Cultures of Chick Embryo Liver Cells

The method has been described in chapter I, A, ii. The results of these experiments are shown in table V.

Discussion

The results obtained with the series of aromatic esters support the hypothesis of Hirsch et al. (1967). Thus, diethyl 2,3,5,6-tetramethylterephthalate (Fig. 12A), the most active porphyrin-inducing compound, has two ester functions sterically hindered from hydrolysis. On the other hand diethyl 2,5-dimethylterephthalate (Fig. 14A) in which the steric hindrance to hydrolysis of the ethoxycarbonyl substituents is largely removed is inactive. Ethyl 2,4,6-trimethylbenzoate (Fig. 12B) which has one sterically hindered ester group has considerable porphyrin-inducing activity whereas ethyl benzoate (Fig. 14E), ethyl 3,5-dimethylbenzoate (Fig. 14D), ethyl 2-(2,4,6-trimethylphenyl)ethanoate (Fig. 14B) and

TABLE V. PORPHYRIN ACCUMULATION IN PRIMARY CULTURE OF CHICK EMBRYO LIVER CELLS INDUCED BY A VARIETY OF CHEMICALS AND MEASURED BY FLUORESCENCE MICROSCOPY

Compound Tested	Concentration		Fluorescence Intensity			
	($\mu\text{g/ml}$)	($\text{m} \times 10^{-5}$)	Expt. 1	Expt. 2	Expt. 3	Mean
Diethyl 2,3,5,6-tetramethylterephthalate	10	3.6	3.5,3,3.5	3,4		3.5
	3	1.1		2.5,2.5,2		2
	1	0.4	1,1	1,0.5		1
Diethyl 2,5-dimethylterephthalate	100	40.0	0,0,0	tr(2),0		0
	10	4.0	0,0,0	0,0,0		0
Ethyl 2,4,6-trimethylbenzoate	50	24.3	2.5,2.5,3	1.5,1,1.5	1.5,2,2	2
	20	9.7	2,1,2.5		1.5,2,1.5	2
	10	4.9		tr(2),1	tr,1,0.5	0.5
Ethyl-2(2,4,6-trimethylphenyl)-ethanoate	50	24.3	tr(3)	1,1.5,1	0,tr,0	0.5
	20	9.7	tr(2),0		0.5,tr(2)	tr
	10	4.9		0,0,0	0,0,0	0
Ethyl-3(2,4,6-trimethylphenyl)-propanoate	100	45.4	0,tr,0	0,tr,0		0
	20	9.1	0,0,0	0,0,0		0
Ethyl-3,5-dimethylbenzoate	100	56	0,tr,0	0,0,0		0
	10	5.6	0,0,0	0,0,0		0
Ethyl benzoate	100	67	0,0,0	0,0,tr		0
	10	6.7	0,0,0	0,0,0		0
2,4,6-Trimethylbenzamide	100	61.4	1.5,2,2	2,2,2		2
	50	30.7	2.5,2.5,2	2,2,2		2
	25	15.3		0.5,tr,0		0.5
	5		0,0			0
2-(2,4,6-Trimethylphenyl)-ethanamide	100	56.5	2,2,1	2,2,2		2
	50	28.3		2,2.5,2.5		2.5
	25	14.1	1.5,1.5,1.5	2,2.5,2.5		1.5
	5	2.8		1.5,1.5,1.5		1.5

TABLE V (continued). PORPHYRIN ACCUMULATION IN PRIMARY CULTURE OF CHICK EMBRYO LIVER CELLS INDUCED BY A VARIETY OF CHEMICALS AND MEASURED BY FLUORESCENCE MICROSCOPY

Compound Tested	Concentration		Fluorescence Intensity			
	($\mu\text{g/ml}$)	($\text{m} \times 10^{-5}$)	Expt. 1	Expt. 2	Expt. 3	Mean
3-(2,4,6-Trimethyl-phenyl)-propanamide	50	26.2	2,2.5, 2.5	2.5,2.5, 2.5		2.5
	25	13.1	2.5,3, 2.5	2,2,2		2
	5	2.6	2,2,2	0.5,1,1		1
3,5-Dimethylbenzamide	100	67	1.5,1.5, 1	0.5,tr,1		1
	10	6.7	0,0,0	0,0,0		0
Benzamide	50	41.3	0,0,0			0
	5	4.1	0,0,0			0

ethyl 3-(2,4,6-trimethylphenyl)propanoate (Fig. 14C) in which the steric hindrance to hydrolysis of the ethoxycarbonyl substituent is removed are inactive. Comparison of the activities of diethyl 2,3,5,6-tetramethylterephthalate (Fig. 12A) and ethyl 2,4,6-trimethylbenzoate (Fig. 12B) suggests that one ethoxycarbonyl group with two ortho-methyl substituents is necessary for activity, while a second ethoxycarbonyl group in the molecule with two ortho-methyl substituents reinforces this activity.

The idea that the activity of 2,4,6-trimethylbenzamide (Fig. 15A) was due to a sterically hindered amide group received support from the inactivity of benzamide (Fig. 15E) and the low activity of 3,5-dimethylbenzamide (Fig. 15D). However, the activity of 2-(2,4,6-trimethylphenyl)ethanamide and 3-(2,4,6-trimethylphenyl)propanamide (Fig. 15C) was not expected since the steric hindrance to hydrolysis of the amide group is removed in these compounds. The possible reasons for this finding are investigated further in Chapter III.

C. Griseofulvin Analogues

Introduction

The structure of griseofulvin (Fig. 17A) and analogues are given in Fig. 17. The systematic nomenclature is based on the trivial name grisan (Fig. 16) for the tricyclic system and according to this nomenclature griseofulvin is designated as 7-chloro-4,6,2'-trimethoxy-6'-methylgris-2'-en-3,4'-dione. In the following discussion the more common names of the analogues will be used where available.

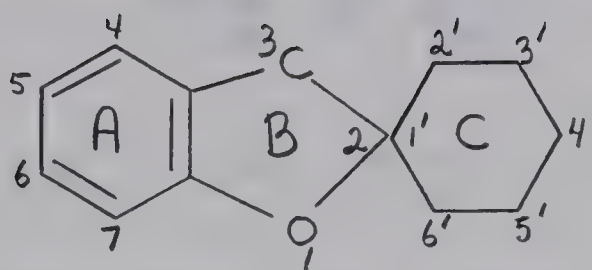
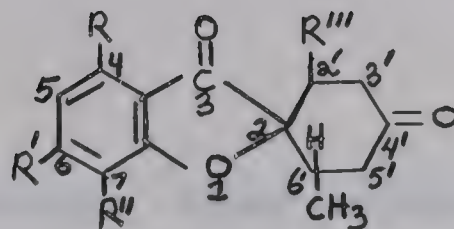


Fig. 16 Grisan

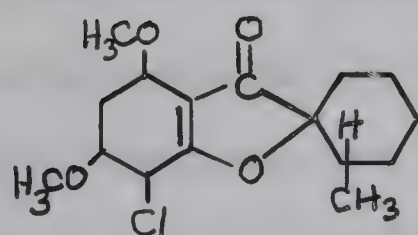
Griseofulvin has two asymmetric carbon atoms which are located at positions 2 and 6'. The configuration at the two asymmetric centers 2 and 6' is designated by d or l. Griseofulvin is prefixed by (d,d), the optical antipode by (l,l), and the diastereoisomer epimeric at position 2 by (l,d); the spiran center (position 2) being the first mentioned.

Granick (1966) compared Courtald models of DDC (Fig. 5A) and griseofulvin (Fig. 17A) and pointed out that it was possible to twist a Courtald model of DDC so that the two oxygen atoms (starred in Fig. 5A) were separated by the same distance as the two oxygen atoms in griseofulvin (starred in Fig. 8C).

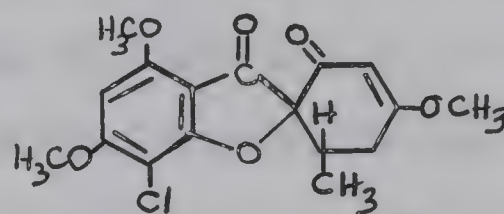
Hirsch (1966) showed that in analogues of griseofulvin (Fig. 17A), namely dechlorogriseofulvin (Fig. 17B), 7-chloro-2'-methoxy-6'-methylgris-2'-en-3,4'-dione (Fig. 17C) and a diastereoisomer of griseofulvin (Fig. 17D) activity was retained. However, activity was found to be reduced in an analogue containing only one keto-oxygen atom, viz. 7-chloro-4,6-dimethoxy-6'-methylgrisan-3-one (Fig. 17I) and in iso-griseofulvin (Fig. 17J) an analogue in which the distance between the



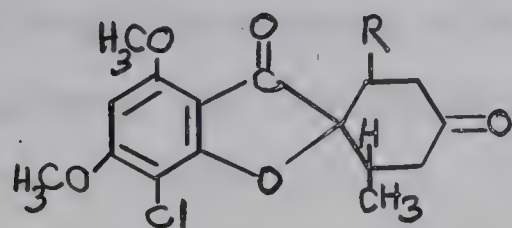
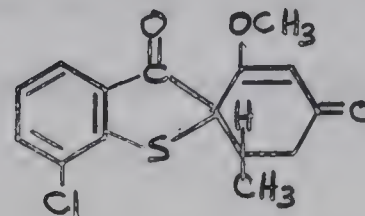
- A. $R = R' = R''' = \text{OCH}_3$; $R'' = \text{Cl}$ (d,d)
 B. $R = R' = R''' = \text{OCH}_3$; $R'' = \text{H}$ (d,d)
 C. $R = R' = \text{H}$; $R''' = \text{OCH}_3$; $R'' = \text{Cl}$ (dd, ll)
 D. $R = R' = R''' = \text{OCH}_3$; $R'' = \text{Cl}$ (l,d)
 E. $R = R' = \text{OCH}_3$; $R''' = \text{H}$; $R'' = \text{Cl}$ (d,d)
 F. $R = R' = R''' = \text{OCH}_3$; $R'' = \text{Br}$ (d,d)
 G. $R = R' = \text{OCH}_3$; $R''' = \text{OC}_2\text{H}_5$; $R'' = \text{Cl}$ (d,d)
 H. $R = R' = \text{OCH}_3$; $R''' = \text{NH}_2$; $R'' = \text{Cl}$ (d,d)



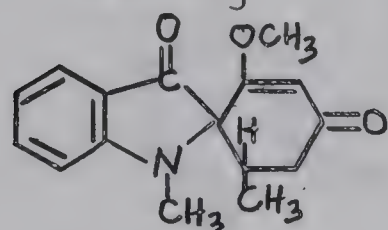
I. (d,d)



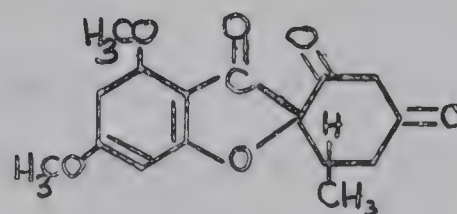
J. (d,d)

K. $R = \text{H}$ (d,d)L. $R = \text{OCH}_3$ (d,d)

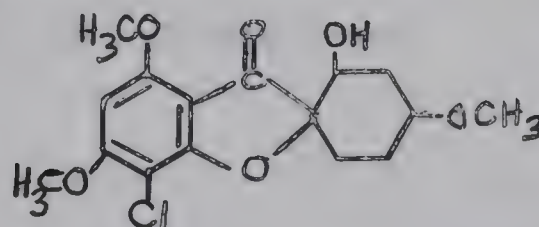
M. (dd, ll)



N. (dd, ll)



O. (d,d)



P. (d,d)

Fig. 17 Chemical Structures of Griseofulvin Analogues

two keto-oxygen atoms is decreased. These findings supported the interpretation of Granick (1966) that the two keto-oxygen atoms and the distance between them were important structural features for activity.

However, the importance of other features in the molecule for optimal activity was revealed by further studies. Thus, the importance of the double bond and methoxy group in ring C was revealed by the reduced activity of desmethoxygriseofulvin (Fig. 17E) and 7-chloro-4,6-dimethoxy-6'-methylgrisan-3,4'-dione (Fig. 17K). Hirsch (1966) pointed out that despite the differences in the porphyria-inducing activity of the griseofulvin analogues, all of the analogues displayed some activity. This finding contrasts with that observed in the terephthalate series where small structural changes resulted in inactivity. It thus appeared that the porphyria-inducing activity was inherent in the grisan-3-one structure (Fig. 18). It was further suggested that the molecular feature

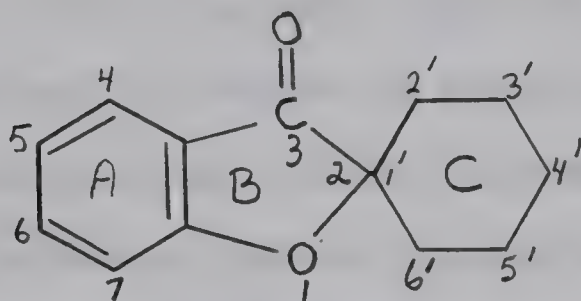


Fig. 18 Grisan-3-one

responsible for activity is the keto group in the 3 position which is maintained in a plane perpendicular to that of ring C because of the stereochemistry of the spiran center 2.

Since further griseofulvin analogues, namely bromogriseofulvin

(Fig. 17F), homogriseofulvin (Fig. 17G), dihydrogriseofulvin (Fig. 17L), the sulfur-containing analogue (Fig. 17M), the nitrogen-containing analogue (Fig. 17N), griseofulvic acid (Fig. 17O), griseofulvamine (Fig. 17H), and tetrahydrogriseofulvin (Fig. 17P) were made available to our laboratory through the courtesy of Dr. T. P. C. Mulholland of Imperial Chemical Industries, Great Britain, we were in a position to carry out further studies on this problem.

Discussion

The compounds (Fig. 17 D, I, E and K) which had been studied by Hirsch (1966) were re-examined and the results obtained were found to be similar to those previously reported. Examination of the compounds, bromogriseofulvin (Fig. 17F), dihydrogriseofulvin (Fig. 17L), homogriseofulvin (Fig. 17G), the sulfur-containing analogue (Fig. 17M), and the nitrogen-containing analogue (Fig. 17N) revealed porphyria-inducing activity. Some of these compounds contain major changes in structure from griseofulvin but all contain the keto group in the 3 position in a plane perpendicular to ring C. Thus, the suggestion of Hirsch (1966) that this is the molecular feature responsible for activity is supported. The reduced activity of homogriseofulvin (Fig. 17G) and dihydrogriseofulvin (Fig. 17L) is in accord with the previous observation of Hirsch (1966) with regard to the importance of the double bond and the methoxy group on ring C for optimal activity. The acidic griseofulvic acid (Fig. 17O) was inactive and the basic griseofulvamine (Fig. 17H) was weakly active, while the neutral analogues of griseofulvin, with the single exception of tetrahydroisogriseofulvin (Fig. 17P) all exhibited

TABLE VI. PORPHYRIN ACCUMULATION IN PRIMARY CULTURE OF CHICK EMBRYO LIVER
CELLS INDUCED BY GRISEOFULVIN ANALOGUES AND MEASURED BY FLUOR-
ESCENCE MICROSCOPY

Compound Tested	Concentration		Intensity of Fluorescence		Mean
	(µg/ml)	(m x 10 ⁻⁵)	Expt. 1	Expt. 2	
17D (Diastereoisomer of griseofulvin)	10	2.8	3,3	3,2.5,3	3
	2	0.6	2,1.5	0.5,2,2	1.5
17I	10	3.1	2,2	1,1.5,0.5	1
	2	1.6	1,1	0.5,1,1	1
17K	10	3.2	0.5,0.5	1,1.5,1	1
	2	0.6	0.5,0.5	0.5,0.5,0.5	0.5
17E (Desmethoxygriseofulvin)	10	3.1	0,0	0,0,0	0
	2	0.6	0.5,0.5	1.5,1,1	1
17F (Bromogriseofulvin)	10	2.5	1,2,2	2.5,1.5	2
	2	0.5	1.0,5,1	0,5,0.5,1	1
17M	10	3.2	2,2,2	2.5,2.5,2	2
	2	0.6		2.5,2.5,2.5	2.5
17N	10	3.7	2,2,2	0.5,1,1	1.5
	2	0.8	2,2,2	1,0.5,0.5	1
17G (Homogriseofulvin)	10	2.7	1,1,0.5	1,1,1	1
	2	0.5	1,1,1	tr,0.5,1	1
17L (Dihydrogriseofulvin)	10	2.8	1,0.5,tr	tr,0.5	0.5
	2	0.6	tr(3)	0,tr(2)	tr
17O (Griseofulvic acid)	10	3.0	0,0,tr	tr(2),0	0
	2	0.6	0,tr,0	0,0,tr	0
17H (Griseofulvamine)	10	3.0	0.5,0,tr	tr(2)	tr
	2	0.6	0,0,tr	0,0,tr	0
17P (Tetrahydrogriseofulvin)	10	2.9	0,0,0	0,0,0	0
	2	0.6	1,tr,tr	0,0,0	0-tr

tr = trace

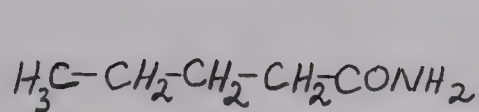
activity. This is probably due to the fact that griseofulvic acid and griseofulvamine exist as charged molecules at physiological pH and would experience difficulty in crossing the cell membrane (Albert, 1965). The inactive analogue tetrahydrogriseofulvin (Fig. 17P) contains a hydroxyl group so that it is probably considerably more water soluble than other derivatives of griseofulvin considered in this thesis. For this reason it is probable that it is less able to cross the cell membrane and this fact might account for its inactivity.

CHAPTER III HYDROLYSIS OF AMIDES AND ESTERS BY CHICK EMBRYO LIVER
AMIDASES AND ESTERASES

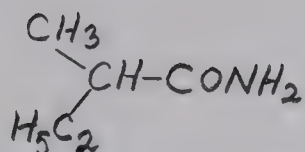
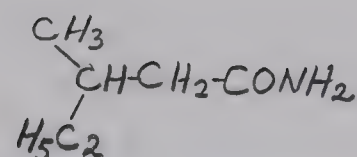
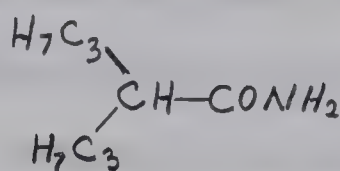
Introduction

Our studies on the relationship between chemical structure and porphyria-inducing activity suggested that in the series of aliphatic and aromatic esters a sterically hindered ester group is a requirement for porphyria-inducing activity. Similarly, in the series of aliphatic amides a sterically hindered amide group is required for activity. On the other hand, in the series of aromatic amides studied, a sterically hindered amide group does not appear to be necessary for activity. In attempting to interpret the above results, the recent work of Granick (1966) is pertinent. This author has suggested that porphyria-inducing drugs are oxidatively metabolized and that excess porphyrin is produced in response to an increased requirement for protoheme. The protoheme formed is thought to be utilized as the prosthetic group of cytochrome p-450 which is required for the oxidative metabolism of the porphyria-inducing drug. This suggested a possible explanation of our results: drugs that cannot be metabolized by a hydrolytic mechanism because of steric factors, are oxidatively metabolized and increased protoheme and porphyrin formation is required. In order to ascertain whether this explanation is correct, one of the factors remaining to be determined is to what extent the "six-number" and the magnitude of the ratio $k^{40^\circ} \text{CH}_3\text{COOH} / k^{40^\circ} \text{RCOOH}$ are indices of the difficulty of enzymic hydrolysis of the compounds studied. For this reason we have conducted preliminary investigations of the rates of hydrolysis of a series of aliphatic amides, a series of aromatic amides, and a series of aliphatic di-esters (Fig. 19) by chick embryo liver amidases and esterases.

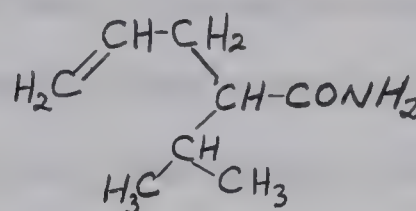
The idea that the action of hydrolytic enzymes is hindered by



n-Valeramide

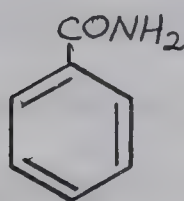
 α -methylbutyramide β -methylbutyramide

Di-n-propylacetamide

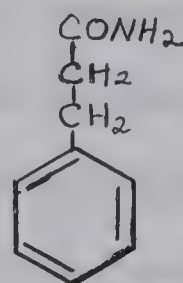


Allylisopropylacetamide

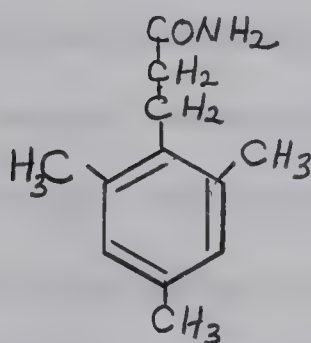
A. ALIPHATIC AMIDES



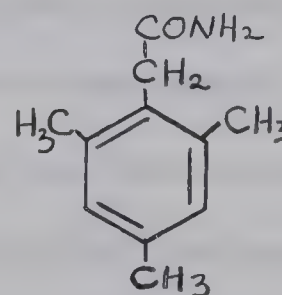
Benzamide



3-Phenylpropanamide

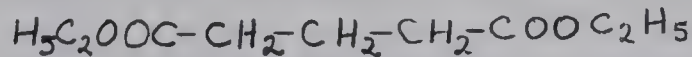


3-(2,4,6-Trimethylphenyl)-propanamide

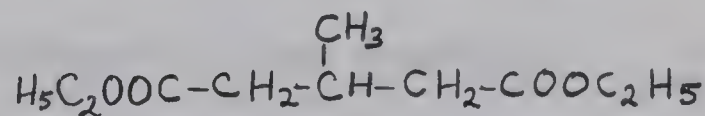
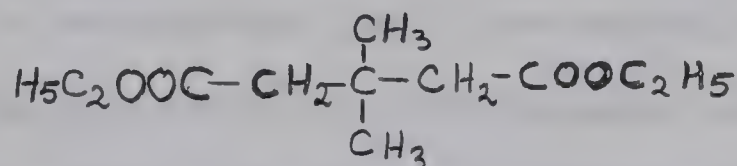


2-(2,4,6-Trimethylphenyl)-ethanamide

B. AROMATIC AMIDES



Diethyl glutarate

Diethyl β -methylglutarateDiethyl β,β -dimethylglutarate

C. ALIPHATIC DIESTERS

Fig. 19 Chemical Structures of Compounds Tested as Substrates
of Chick Embryo Liver Amidase and Esterase

steric factors in substrate molecules receives support from the following considerations. Many similar models have been proposed to explain the mechanism of ester hydrolysis by esterases and that shown in Fig. 20 contains the essential features of current ideas (Westheimer, 1962).

In this mechanism the imidazole functions as a general base catalytic agent since it attracts the serine hydroxyl hydrogen and thus increases the nucleophilicity of the serine hydroxyl oxygen atom. This oxygen atom attacks the carbonyl carbon of the ester and the acylated enzyme intermediate III is formed. An acidic group facilitates rupture of the acyl oxygen bond of the ester by functioning as a general acid catalytic species as shown in the figure. Deacylation of the enzyme is accomplished by nucleophilic attack of water at the carbonyl carbon of the acylated enzyme intermediate III forming the intermediate IV which subsequently breaks down forming the free enzyme and acid as shown in V. It seems reasonable that such a mechanism should be sensitive to hindrance by steric factors in an ester molecule since both acylation and deacylation depend upon nucleophilic attack at a carbonyl carbon atom. Shielding of the carbonyl carbon by bulky groups in close proximity would hinder the approach of a nucleophile and thus reduce the rate of reaction. Recently Fife and Milstein (1967) have studied the effect of steric factors in the deacylation of acyl-chymotrypsins formed from a series of p-nitrophenyl esters. These workers found that increasing steric bulk in the acyl group leads to decreased rates of deacylation. Thus propionyl-chymotrypsin is deacylated much more rapidly than trimethylacetyl- or 3,3-dimethylbutyrylchymotrypsin. Acylation of the enzyme could not be achieved when p-nitrophenyl triethylacetate was

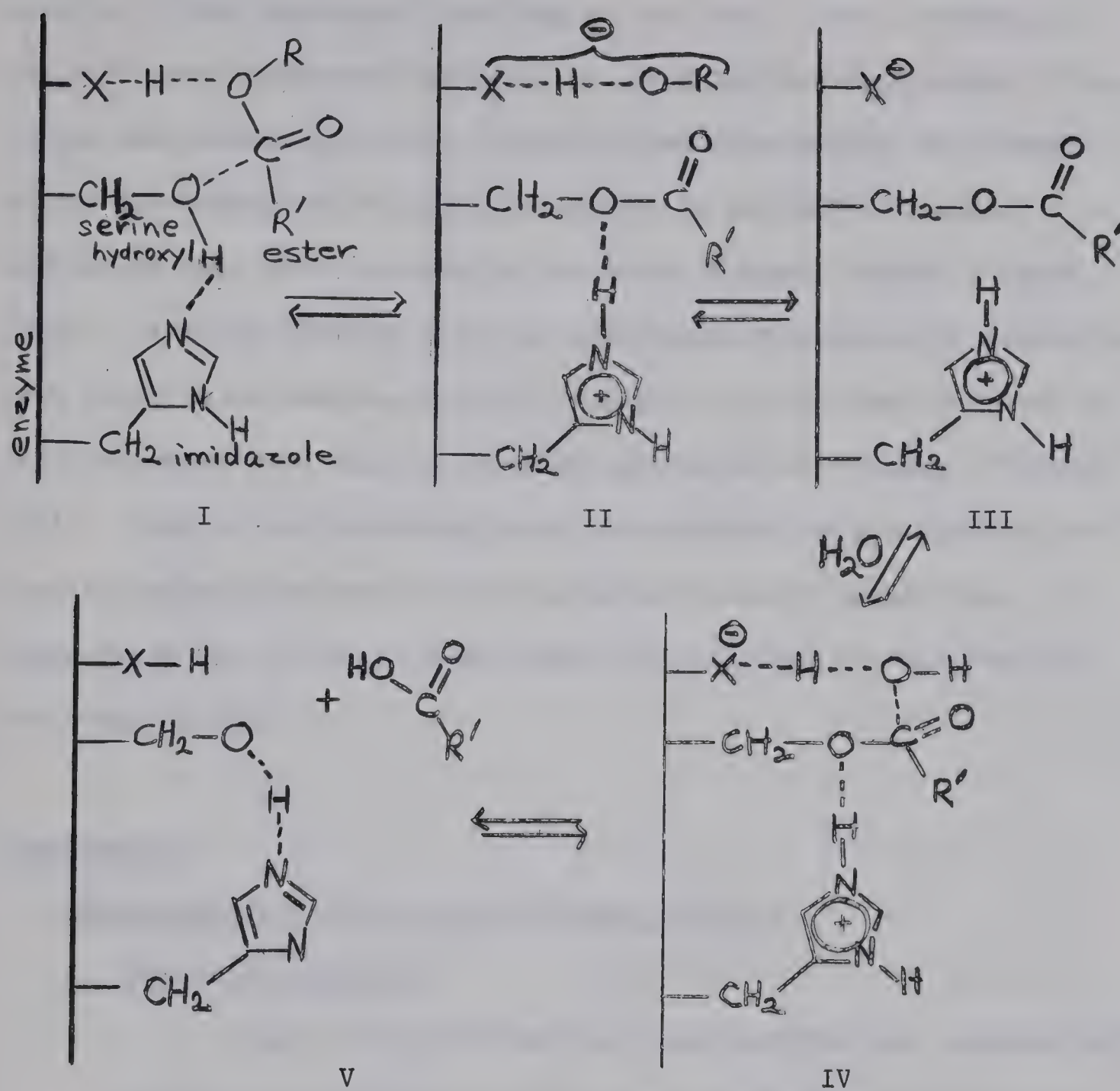


Fig. 20 Proposed Mechanism of the Hydrolysis of Esters by
Esterase

employed. Thus increased branching at both the α and β carbons of the acyl group decreased the rates of acylation and deacylation. Since it has been established that an acyl enzyme intermediate is formed during the hydrolysis of amide substrates it follows that amide hydrolysis must also be sensitive to steric factors (Bender & Kézdy, 1964). It is of interest that the hydrolysis of a series of esters has been found to be more susceptible to steric factors when catalyzed by cholinesterase than when catalyzed by hydroxide ion (Thomas & Stoker, 1961). This is not surprising when one compares the possibilities for steric hindrance encountered by the serine hydroxyl oxygen when approaching the carbonyl carbon (Fig. 20) with that of a hydroxide ion (Chapter IIA).

Experimental

i. Determination of Chick Liver Amidase Activity

a. Enzyme Preparation

Livers from 17-19 day old chick embryos were removed and cooled on ice after which they were homogenized in ice cold 1.15% KCl (4 ml/g wet wt liver). In our initial experiments 1 ml of this homogenate was used and this is referred to as the "crude amidase" preparation. In our subsequent studies the cold homogenate was centrifuged at 9,000 g and 4° for 20 minutes. The supernatant (1 ml) which retained all the activity of the homogenate, was used and is referred to as the "amidase" preparation to distinguish it from the earlier crude extract.

b. Assay for Amidase Activity

Amidase activity of the "crude amidase" or "amidase" preparations was determined by measuring the amount of ammonia released from a series of amides. A microdiffusion apparatus (Fig. 21) developed by Conway and modified by Öbrink (1955, 1962) was used for this purpose. To the closing chamber was added saturated K_2CO_3 (1.5 ml) and a few drops of a nonionic neutral wetting agent (0.025%; NPX Tergitol^R) to facilitate dispersion of the K_2CO_3 solution over the polypropylene surface. A boric acid solution (1 ml; 1%), prepared as described below, and a few drops of tergitol was added to the inner chamber. The ammonia content of a particular solution was determined by adding the solution (1 or 2 ml) to the outer chamber of the microdiffusion apparatus. Saturated K_2CO_3 (1 ml) was added rapidly to the outer chamber and the lid quickly placed in the closing chamber. The liquid seal formed between the lid and fluid in the closing chamber prevented any loss of ammonia. The ammonia gas evolved following addition of the K_2CO_3 solution diffuses into the boric acid solution in the inner chamber and is converted into NH_4^+ ions according to the following equation:



The boric acid solution changes in color from a faint red to green. Following a suitable ammonia diffusion time (Conway, 1962) the $H_2BO_3^-$ ions formed are titrated with N/50 HCl using a microsyringe (Roger Gilmont Instruments, Inc.). The end point of

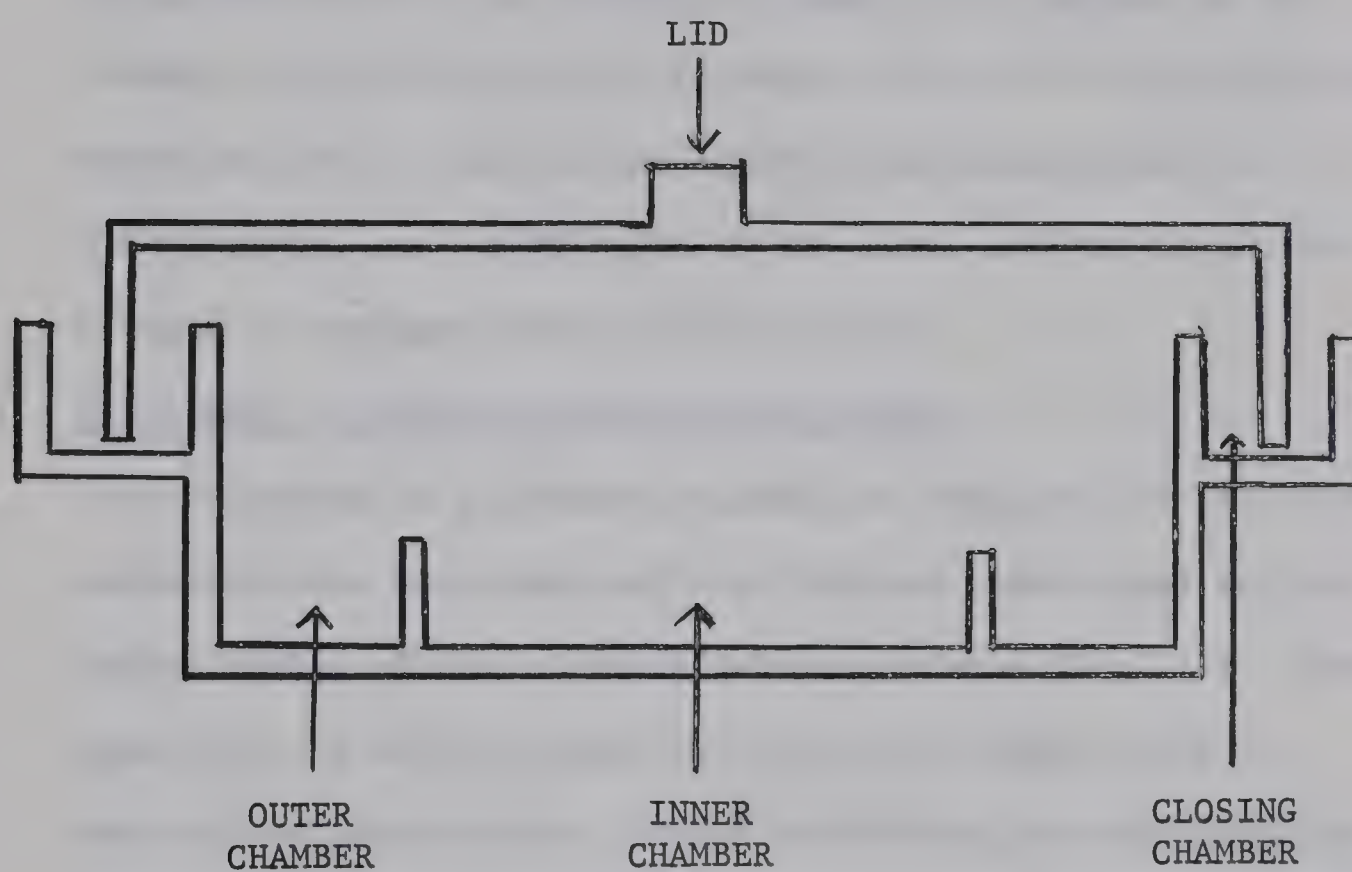


Fig. 21 Microdiffusion Dish Used to Measure Amidase Activity

the titration is determined by the transition of the green boric acid solution to a faint red (pH 5.0-5.1). The number of equivalents of NH_4^+ ions trapped in the boric acid solution equals the number of equivalents of N/50 HCl added.

The boric acid solution was made as follows: Boric acid (10 g) was added to a volumetric flask (1 l) containing 98% ethanol (200 ml) and distilled water (700 ml). A mixed indicator solution (10 ml) composed of 0.033% bromocresol green and 0.066% methyl red in 98% ethanol was added and the flask was brought to the mark with distilled water.

c. Efficiency of the Microdiffusion Apparatus

Solutions of a primary standard of $(\text{NH}_4)_2\text{SO}_4$ of different concentrations were made and 1 ml aliquots were added to the outer chamber of the microdiffusion apparatus (Fig. 21). Saturated K_2CO_3 (1 ml) was added to the outer chamber and the ammonia gas released was allowed to diffuse into the boric acid solution in the inner chamber for 3 hours. The amount of NH_4^+ trapped in the boric acid solution was determined by titration with N/50 HCl. The results of this experiment are shown in table VII.

d. Study of the Efficiency of the Microdiffusion Apparatus for Recovery of NH_4^+ Ions Added to the "Crude Amidase" Preparation

Solutions of $(\text{NH}_4)_2\text{SO}_4$ (0.5 ml) of different concentrations were added to a mixture of "crude amidase" preparation (1 ml) and 0.5 M potassium phosphate buffer, pH 7.2 (0.5 ml) in the outer chamber of the microdiffusion apparatus. Saturated

K_2CO_3 (1 ml) was added to the outer chamber and the ammonia gas released was allowed to diffuse into the boric acid solution for 3 hours. The amount of NH_4^+ trapped in the boric acid solution was determined by titration with N/50 HCl. The results of this experiment are shown in table VIII.

e. Hydrolysis of n-Valeramide by "Amidase" Preparation

Solutions of n-valeramide (0.5 ml) of different concentrations were added to a mixture of "amidase" preparation (1 ml) and 0.5 M potassium phosphate buffer, pH 7.9 (0.5 ml) in test tubes and the mixtures were incubated at 37° for 6.5 hours. The contents of the test tubes were then transferred to the outer chamber of the microdiffusion apparatus and the ammonia content of the mixtures was determined as previously described. The results of this experiment are shown in table IX.

f. The Effect of pH on the Enzymic Hydrolysis of n-Valeramide

Solutions of n-valeramide theroretically capable of liberating 276 μg NH_4^+ were added (0.5 ml) to a mixture of "amidase" preparation (1 ml) and 1 M potassium phosphate buffer (1 ml) at varying pH values. Following incubation at 37° for 6.5 hours the ammonia content of the mixtures was determined as previously described. The results of this experiment are shown in figure 22.

g. Time Course of the Enzymic Hydrolysis of n-Valeramide

Solutions of n-valeramide (0.5 ml) theoretically capable of liberating 276 μg NH_4^+ were added to a mixture of "amidase" preparation (1 ml) and 1 M potassium phosphate buffer, pH 9.6

(0.5 ml) in test tubes and the mixtures were incubated for varying time intervals at 37°. The ammonia content of the mixtures was then determined as previously described. The results of this experiment are shown in figure 23.

h. Hydrolysis of a Series of Aliphatic Amides by the "Amidase"

Preparation

Solutions of the aliphatic amides (17 μ m/0.5 ml) theoretically capable of liberating 306 μ g NH_4^+ were made and added in 0.5 ml aliquots to mixtures of "amidase" preparation (1 ml) and 1 M potassium phosphate buffer, pH 9.6 (0.5 ml) in test tubes. Following incubation for 7 hours at 37° the ammonia content of the mixtures was determined as previously described. The results of this experiment are shown in table X.

i. Hydrolysis of a Series of Aromatic Amides by the "Amidase"

Preparation

Solutions of the aromatic amides (15 μ m/0.5 ml) theoretically capable of liberating 270 μ g NH_4^+ were made and added in 0.5 ml aliquots to a mixture of "amidase" preparation (1 ml) and 1 M potassium phosphate buffer, pH 9.6 (0.5 ml) in test tubes. Following incubation for 5 hours at 37° the ammonia content of the mixtures was determined as previously described. The results of these experiments are shown in table XI.

ii. Determination of Chick Liver Esterase Activity

a. Enzyme Preparation

Livers from 17-19 day old chick embryos were removed and

TABLE VII. STUDY OF THE EFFICIENCY OF THE MICRODIFFUSION APPARATUS

Amount of NH_4^+ Added (μg)	Volume of N/50 HCl Required for Titration (ml)	Net NH_4^{+*} Recovered (μg)	Percentage Recovery
642.4	1.577	565.0	88
642.4	1.570	562.3	
321.2	0.827	294.8	92
321.2	0.830	295.9	
160.6	0.433	153.0	95
160.6	0.432	153.0	
80.3	0.216	74.9	95
80.3	0.224	77.8	
40.2	0.118	39.6	97
40.2	0.114	38.2	
Distilled	0.008	2.88	
Water	0.008	2.88	
(1 ml)			

* The net NH_4^+ recovered was determined by subtracting the amount of ammonia detected in 1 ml of distilled water, viz., 2.88 μg , from the value obtained from the titration data. The mean of duplicate samples is shown.

TABLE VIII. STUDY OF THE EFFICIENCY OF THE MICRODIFFUSION APPARATUS FOR
RECOVERY OF NH_4^+ IONS ADDED TO "CRUDE AMIDASE" PREPARATION

Amount of NH_4^+ Added to "Crude Amidase" (1 ml) and Potassium Phosphate Buffer (0.5 ml) (μg)	Volume of N/50 HCl Required for Titration (ml)	Percentage* Recovery
160.6	0.457	91.0
160.6	0.459	
80.3	0.256	94.0
80.3	0.256	
40.2	0.151	90.0
40.2	0.150	
0	0.048	
	0.054	

* The net NH_4^+ recovered was determined by subtracting the amount of ammonia detected in a mixture of "crude amidase" preparation (1 ml) and 0.5 M potassium phosphate buffer, pH = 7.2 (1 ml) and is expressed as the mean of duplicate samples.

TABLE IX. HYDROLYSIS OF n-VALERAMIDE BY "AMIDASE" PREPARATION

Theoretical Amount of NH_4^+ Releasable from Solutions of n-Valeramide (0.5 ml) Added to "Amidase" Preparation (1 ml) and Potassium Buffer (0.5 ml)	Volume of N/50 HCl Required for Titration	Net NH_4^{+*} Recovered
(μg)	(ml)	(μg)
262.0	0.327	63.2
262.0	0.369	
131.0	0.271	36.4
131.0	0.276	
65.5	0.258	31.8
65.5	0.265	
0	0.165	
0	0.180	

* The net NH_4^+ recovered was determined by subtracting the amount of ammonia detected in a mixture of "amidase" preparation (1 ml) and 0.5 M potassium phosphate buffer, pH = 7.9 (1 ml) and is expressed as the mean of duplicate samples. No hydrolysis of a solution of n-valeramide (1 ml) theoretically capable of liberating 262 μg NH_4^+ was observed when incubated with 0.5 M potassium phosphate buffer, pH = 7.9 (1 ml) for 6.5 hours at 37°. Boiling the "amidase" preparation (1 ml) for 25 minutes prior to incubation resulted in complete loss of enzymic activity.

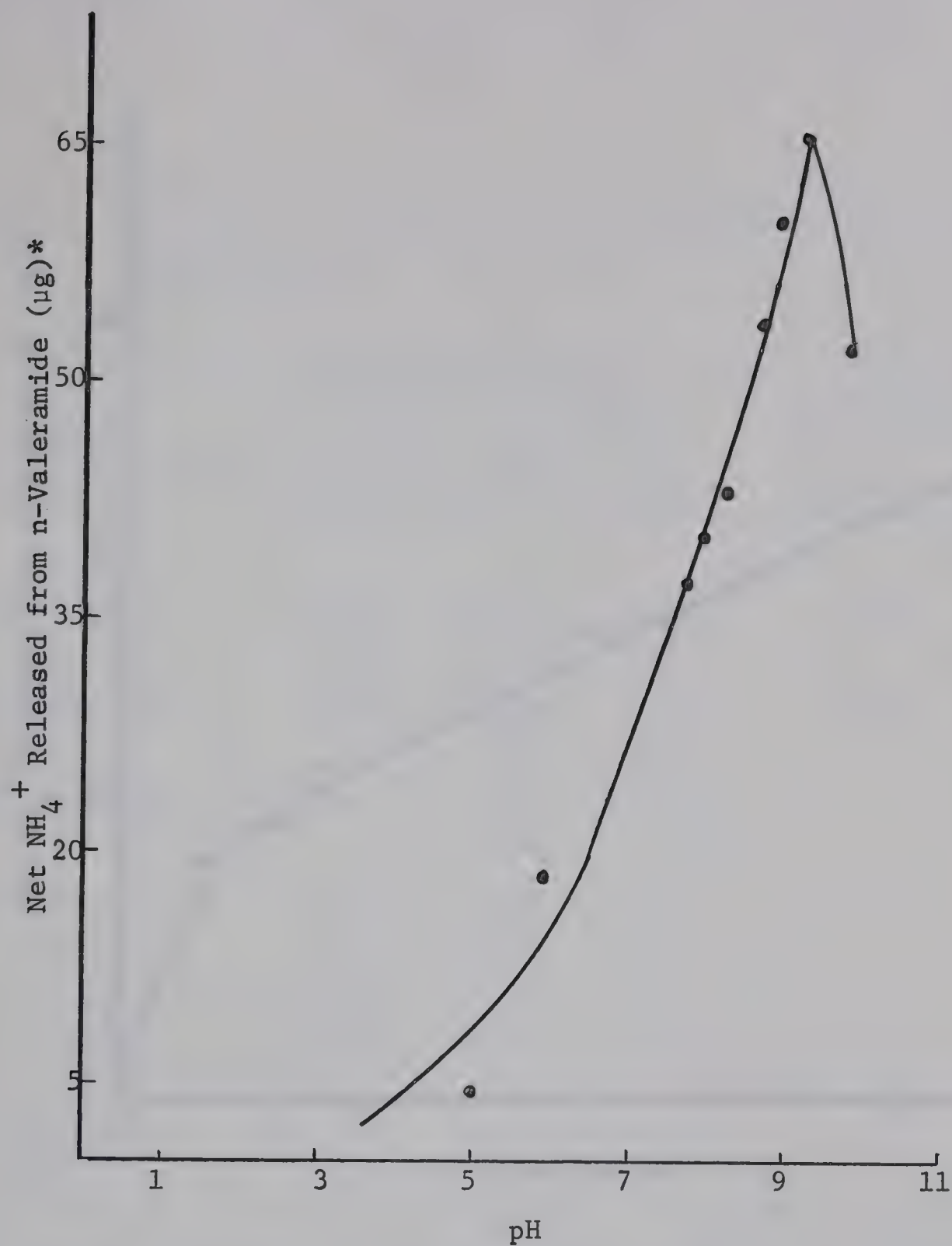


Fig. 22 The Effect of pH on the Enzymic Hydrolysis of n-Valeramide

* The net NH_4^+ released from n-valeramide at each pH value was determined by subtracting the ammonia content in a mixture of "amidase" preparation (1 ml) and 1 M potassium phosphate buffer (1 ml) at the same pH. Each point represents the mean of duplicate samples.

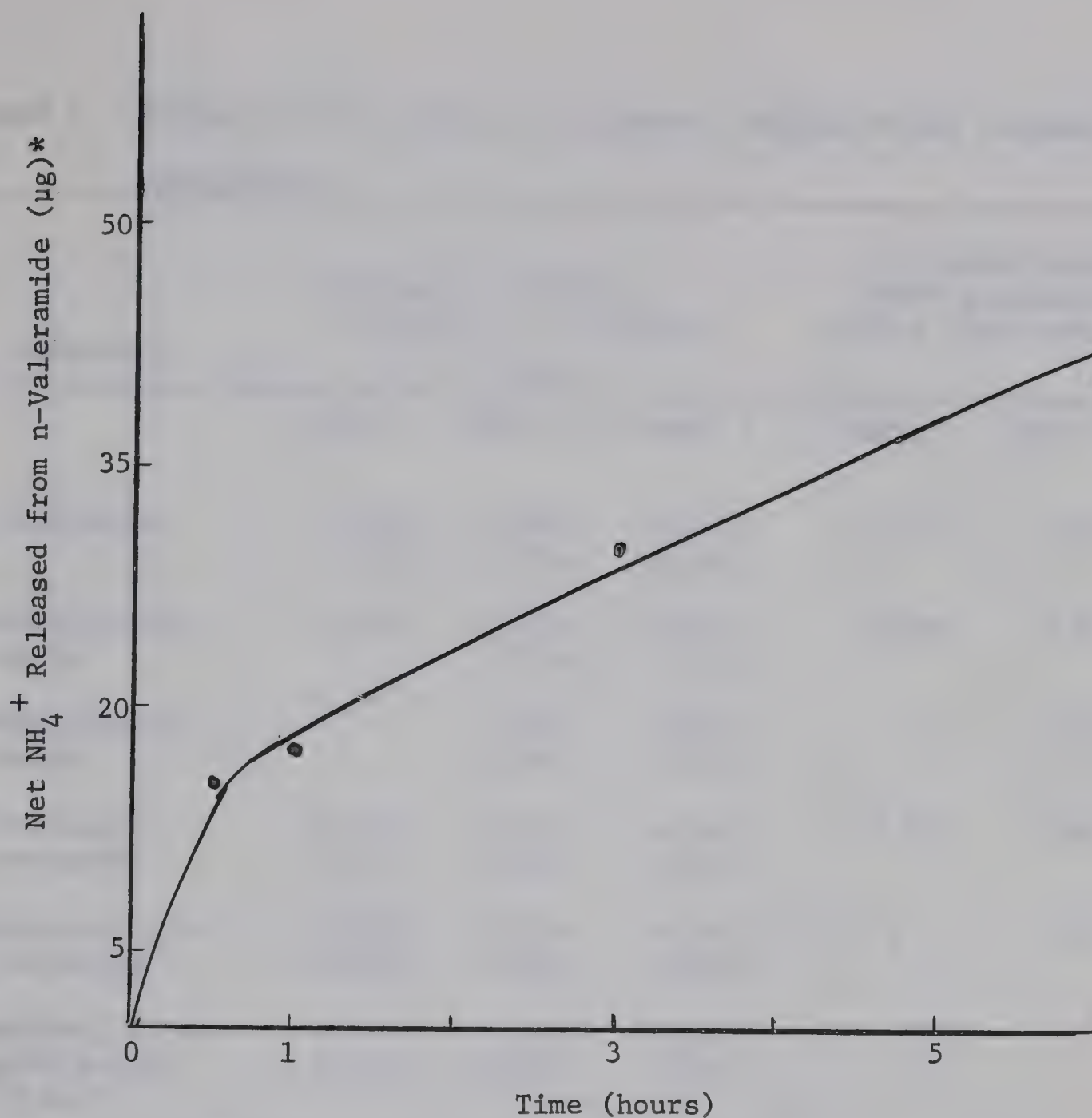


Fig. 23 Time Course of the Enzymic Hydrolysis of n-Valeramide

* The net NH_4^+ released from n-valeramide was determined by subtracting the amount of ammonia detected in a mixture of "amidase" preparation (1 ml) and 1 M potassium phosphate buffer (1 ml). Each point represents the mean of duplicate samples.

TABLE X. HYDROLYSIS OF A SERIES OF ALIPHATIC AMIDES BY THE "AMIDASE"

Substrate	PREPARATION			μ m Amide Hydrolyzed per 9000 g Supernatant from 0.21 g Liver per 7 hours *		
	Volume of N/50 HCl Required for Titration					
	(ml)					
	Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 2	Expt. 3
n-Valeramide	0.336 0.341	0.372 0.384	0.384 0.367	4.56	5.16	4.74
α -Methylbutyr- amide	0.240	0.250 0.250	0.246 0.252	2.59	2.60	2.21
β -Methylbutyr- amide		0.194 0.183	0.200 0.192		1.37	1.12
Di-n-propyl- acetamide	0.200 0.202	0.208 0.227	0.260 0.252	1.81	1.95	2.35
Allylisopropyl- acetamide	0.110 0.117	0.134 0.136	0.124 0.120	0	0.30	0
"Amidase" (1 ml) plus buffer (2 ml)	0.168 0.113	0.116 0.124	0.132 0.145			

* Expressed as the mean of duplicates

TABLE XI. HYDROLYSIS OF A SERIES OF AROMATIC AMIDES BY "AMIDASE" PREPARATION

Substrate	Volume N/50 HCl Required for Titration (ml)			μm Amide Hydrolyzed per 9000 g Supernatant from 0.21 g Liver per 5 hours		
	Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 2	Expt. 3
3-Phenyl propanamide	0.382 0.424	0.204 0.206	0.284 0.276	5.76	2.34	3.81
3-(2,4,6-Trimethyl- phenyl)-propanamide [†]		0.168 0.110	0.135 0.126		0.42 0.26	0.82 0.03
2-(2,4,6-Trimethyl- phenyl)-ethanamide [†]		0.084 0.118	0.088 0.094		0.26	0.03
3,5-Dimethylbenzamide [†]		0.082 0.084			0	
Benzamide	0.118 0.134				0.01	
"Amidase" (1 ml) plus Buffer (1 ml)	0.112 0.128	0.085 0.091	0.087 0.092			

[†] These substrates were poorly soluble in water.

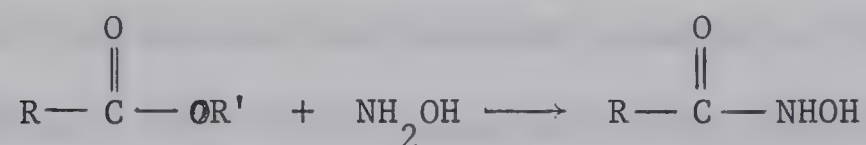
* Expressed as the mean of duplicates.

cooled on ice after which they were homogenized in ice cold 1.15% KCl (5 ml/g wet wt liver). This homogenate (0.5 ml) was used as the esterase preparation.

b. Assay for Esterase Activity

1. Principle of the Assay Method

The amount of hydrolysis of a series of esters by the esterase preparation was determined by measuring the amount of ester remaining after incubation. The colorimetric method developed by Hestrin (1949) and modified by Lee & Levitt (1967) was used for this purpose. In this method an ester is reacted with hydroxylamine to form an hydroxamate according to the following equation:



The hydroxamate formed is treated with FeCl_3 in the presence of acid to form a brown colored complex. The absorbency of this complex is determined at 540 mu. The intensity of absorption is proportional to the amount of ester reacting with hydroxylamine.

2. Reagents Used

Alkaline Hydroxylamine Reagent

Equal volumes of cold 2 M hydroxylamine hydrochloride and 3.5 N sodium hydroxide were mixed and the resulting solution used within 3 hours of preparation.

Ferric Chloride Reagent

A solution of 0.37 M $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 0.1 N HCl was prepared.
4 N HCl

Ester Solutions

Solutions of esters of varying concentrations were prepared in distilled water: 95% ethanol (9 : 1 v/v) and used as described below.

3. Procedure

Formation of the Colored Complex

Alkaline hydroxylamine reagent (2 ml) was added to test tubes containing the ester solutions (1 ml). One ml of 4 N HCl was added followed by the FeCl_3 reagent (1 ml) and the absorbency of the colored complex was measured promptly at 540 mu.

Measurement of the Activity of the Esterase Preparation

An ester solution (1 ml) was added to a mixture of esterase preparation derived from 100 mg of chick embryo liver (0.5 ml) and 0.1 M potassium phosphate buffer, pH 7.2 (0.5 ml) in test tubes. The mixtures were incubated at 37° for 45 minutes. Following incubation alkaline hydroxylamine reagent was added (4 ml) and the hydroxamate was allowed to form over a 10-30 minute period at room temperature. Two ml of 4 N HCl were added and the mixtures were centrifuged to remove the precipitated protein. To the clear supernatant solution was added 2 ml of Fe Cl_3 reagent and the absorbency of the brown colored complex was read at 540 mu.

c. Time Required for Completion of the Reaction Between Aliphatic Di-esters and Hydroxylamine Reagent

Solutions of different concentrations of diethyl glutarate and diethyl β,β -dimethylglutarate were made and the esters were exposed to alkaline hydroxylamine reagent for varying time periods. The amount of hydroxamate formed after these time periods was measured by conversion of the hydroxamate to the brown colored complex as previously described. The results of this experiment are shown in table XII.

d. Investigation of Relationship Between Absorbency and Concentration of Ester

The results obtained above were used to prepare Figs. 24A & 24B which demonstrate the relationship between absorbency and concentration of ester. In addition similar studies were carried out with diethyl β -methylglutarate which are plotted in Fig. 24C.

e. The Hydrolysis of Diethyl glutarate, Diethyl β -methylglutarate, and Diethyl β,β -dimethylglutarate by Chick Embryo Liver Esterase Preparation

Solutions of different concentrations of the above esters were made and 1 ml aliquots of these solutions were added to a mixture of esterase preparation (0.5 ml) and 0.1 M potassium phosphate buffer, pH 7.2 (0.5 ml) in test tubes. To several of these test tubes, cooled on ice, alkaline hydroxylamine reagent (4 ml) was immediately added and after allowing hydroxamate formation to come to completion at room temperature for 30 minutes

the amount of hydroxamate formed was determined as previously described. This procedure is referred to in table XIII as the standard treatment. The remaining test tubes were allowed to incubate for 45 minutes at 37° and the amount of ester remaining was determined as previously described. This procedure is referred to in table XIII as the enzymic treatment.

Discussion

The results shown in tables VII & VIII show that the micro-diffusion apparatus is useful in measuring the amount of ammonia present in solutions and in the "crude amidase" preparation. The recovery of ammonia exceeded 90% which was adequate for our subsequent studies. Very few studies of liver amidases have been carried out previously. Bray (1950) demonstrated the presence of an amidase in rabbit liver and showed that n-valeramide was one of the best substrates for this enzyme. For this reason we selected n-valeramide as a substrate for checking whether an amidase could be detected in chick embryo liver. This amide was shown to be inactive as a porphyria-inducing drug at a concentration of 175 µg/ml using the petri dish technique. The results shown in table IX demonstrate the presence of an amidase in chick embryo liver. Bray (1950) studied the effect of pH on the rabbit liver amidase and showed that the enzyme had optimal activity at pH 8.4. In our studies (Fig. 22) the activity of the enzyme rose as the pH was increased from pH 5, reaching an apparent maximum at pH 9.2. At higher pH values the activity appeared to drop although this could not be established with certainty using phosphate buffer. In all subsequent experiments therefore a pH of 9.2

TABLE XII. TIME REQUIREMENT FOR COMPLETION OF REACTION BETWEEN HYDROXYLAMINE AND ESTERS

Substrate	Amount of Substrate Added (μm)	Net Absorbency after Exposure of Ester to Hydroxylamine Reagent for: *		
		2 minutes	10 minutes	30 minutes
Diethylglutarate	4	1.214	1.194	
		1.234	1.224	
	2	0.608	0.602	
		0.584	0.599	
	1	0.317	0.345	
		0.309	0.299	
	0.5	0.141	0.146	
		0.148	0.139	
Diethyl β,β -dimethylglutarate	3.83	0.792	1.050	1.050
		0.792	1.030	1.030
	1.91	0.399	0.531	0.511
		0.399		0.504
	0.96	0.217	0.237	0.248
		0.223		0.249
	0.48	0.102	0.152	0.147
		0.112	0.130	0.157

* The net absorbency was determined by subtracting the non-specific color of a blank solution prepared as follows: to the ester solution (1 ml) was added 4 N HCl (1 ml) followed by alkaline hydroxylamine reagent (2 ml) and FeCl_3 reagent (1 ml) and the absorbency of this solution is measured. With this order of addition of the various reagents, esters do not form any hydroxamic acid.

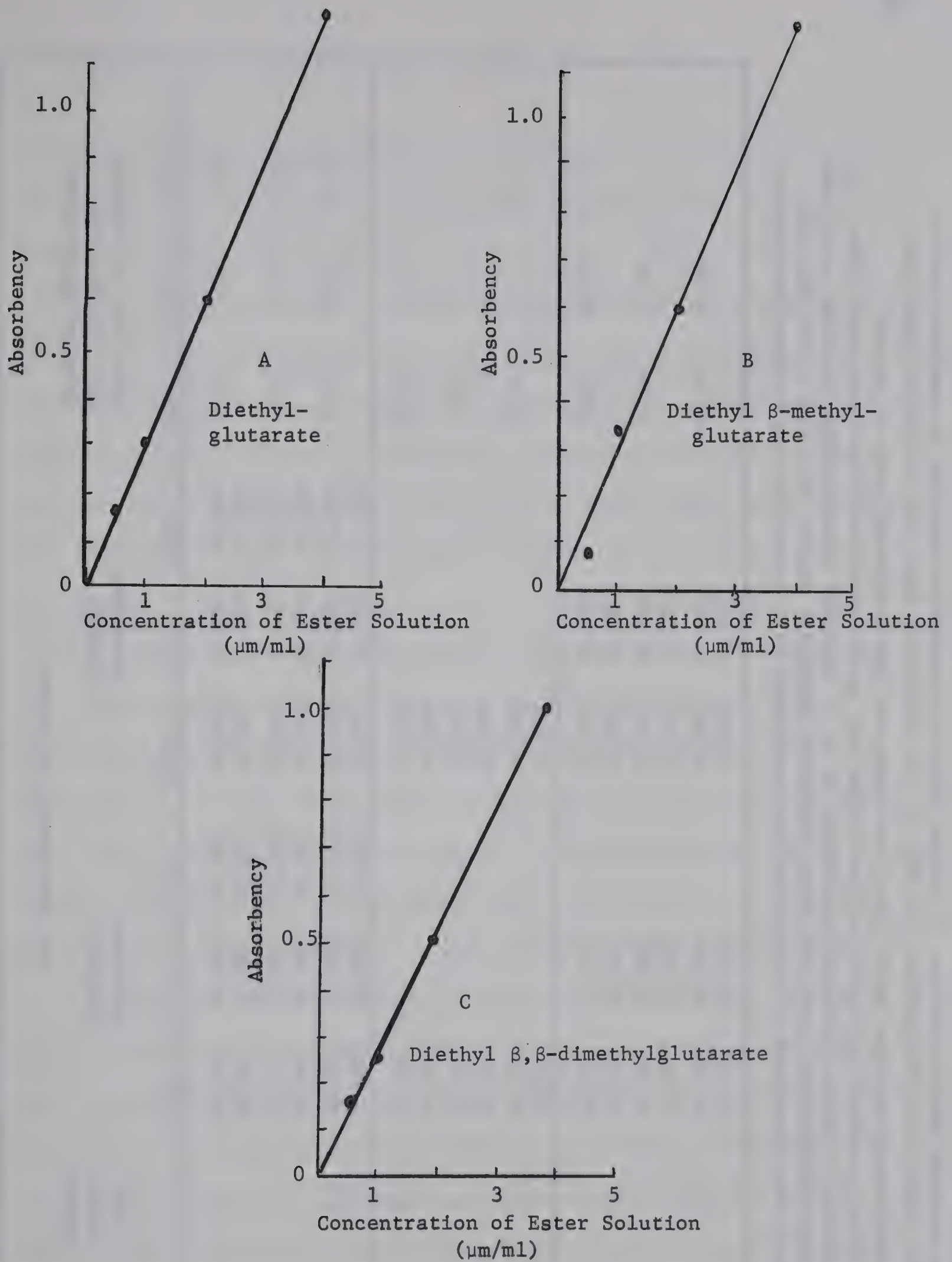


Fig. 24 Linear Relationship Between Absorbency and Concentration of Various Ester Solutions

TABLE XIII HYDROLYSIS OF A SERIES OF ALIPHATIC DIESTERS BY CHICK LIVER ESTERASE PREPARATION.

Substrate	Amount Added (μm)	Net Absorbency Following: *									μm Ester Hydrolyzed per 100 mg wet wt Liver per 45 minutes		
		Standard Treatment			Enzymic Treatment								
		Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 2	Expt. 3			
Diethyl glutarate	4	0.592	0.568	0.502	0.272	0.226	0.235	2.51	3.01	2.58			
		0.505	0.600	0.504	0.252	0.207	0.229						
	2	0.316	0.305	0.248	0.146	0.108	0.137	1.24	1.48	1.03			
		0.296	0.285	0.237	0.152	0.119	0.139						
	1	0.174	0.156	0.124	0.105	0.088	0.035	0.50	0.84	0.37			
		0.168	0.205	0.125	0.094	0.050	0.036						
	4	0.551			0.279			2.22					
		0.562			0.318								
Diethyl β-methyl-glutarate	2	0.275			0.190			0.95					
		0.332			0.178								
	1	0.170			0.120			0.31					
		0.105			0.130								
Diethyl β,β-dimethyl-glutarate	4	0.508	0.596		0.376	0.450		1.07	0.55				
		0.478	0.512		0.390	0.494							
	2	0.246	0.291		0.228	0.245		0.30	0.29				
		0.205	0.256		0.220	0.263							
	1	0.130	0.184		0.095	0.122		0.32	0.34				
		0.126	0.176		0.092	0.135							

* Net absorbency was determined by subtracting the absorbency of a blank solution prepared as follows: To the esterase preparation (1 ml) was added potassium phosphate buffer, pH - 7.2 (1 ml) and the resulting mixture allowed to incubate 45 minutes at 37°. Alkaline hydroxylamine solution (4 ml) was added and after 20 minutes 4 N HCl (2 ml) was added and the protein precipitate was centrifuged. To the clear supernatant solution FeCl₃ (2 ml) was added and the absorbancy read at 540 mu.

No non-enzyme hydrolysis was observed for any of the substrates under the conditions described. Boiling the esterase preparation for 25 minutes prior to incubation resulted in complete loss of enzymic activity.

was used. No significant non-enzymic hydrolysis was noted at this pH. The amount of enzymic hydrolysis of n-valeramide at different time periods is recorded in figure 23.

It was anticipated that the porphyria-inducing amides, AIA and di-n-propylacetamide, would be hydrolyzed with difficulty by the amidase while the inactive compounds, n-valeramide, α -methylbutyramide, and β -methylbutyramide were expected to be readily hydrolyzed. The results with four of the five compounds were in accordance with expectation. However, the ease of hydrolysis of di-n-propylacetamide was not anticipated.

Bray (1950) demonstrated that 3-phenylpropanamide (Fig. 19B) was a good substrate for an amidase prepared from rabbit liver. It was therefore anticipated that 3-phenylpropanamide would be readily hydrolyzed by the "amidase" preparation derived from chick embryo liver and this was found to be true (table XI). This compound was shown to be active as a porphyria-inducing compound (+3, 250 μ g/ml; +1, 100 μ g/ml; tr, 50 μ g/ml) employing the petri dish technique for culturing liver cells. Thus the steric hindrance hypothesis of Hirsch et al. (1967) would appear to have little validity when applied to aromatic amides. This conclusion was strengthened by the observation that benzamide was not hydrolyzed (table XI) by the "amidase" preparation. Benzamide was shown to be inactive as a porphyria-inducing drug (table v) and thus one would anticipate ready hydrolysis of this compound. No firm conclusions could be made concerning the enzymic hydrolysis of the other aromatic amides (table (XI) because of their poor solubility in water.

Hestrin (1949) developed a rapid micromethod for the estimation

of short chain carboxylic acid esters based on their ability to react with hydroxylamine quantitatively in an aqueous alkaline solution. The method was shown to be useful for determination of acetylcholine in the presence of a large excess of acetate and choline. This assay has been modified by Lee & Levitt (1967) and used for estimating the concentration of local anesthetic esters such as procaine. The assay was utilized for investigating the enzymic hydrolysis of these local anesthetic esters. The principal modification employed by Lee & Levitt was to increase the time allowed for hydroxamate formation. Thus in the case of procaine 10 minutes was required for maximum hydroxamate formation at room temperature which was considerably greater than that required by acetylcholine. In our experiments maximum hydroxamate formation was found in a period of 2 minutes with diethyl glutarate and 10 minutes for diethyl β,β -dimethylglutarate (table XII). For this reason in our studies a period of at least 10 minutes was used for hydroxamate formation. Studies shown in table XII and Fig. 24 show that there is a linear relationship between absorbency and concentration of the di-esters.

It was anticipated that the difficulty of hydrolysis of these di-esters would parallel their porphyria-inducing activity, viz., diethyl β,β -dimethylglutarate > diethyl β -methylglutarate > diethyl glutarate. The results shown in table XIII are in accordance with this expectation.

In summary it appears that although steric factors play some part in the biological activity of the series of compounds tested, these factors do not fully explain the activities of the compounds. It appears

that other factors such as the electronic and hydrophobic properties of the molecules may play a part. In this connection it is of interest that Hansch & Deutsch (1966) have had considerable success in relating the physico-chemical properties of drugs with their biological activities by taking into account steric, electronic and hydrophobic properties of drugs. It is likely that further studies of porphyria-inducing compounds which take into account electronic and hydrophobic properties as well as steric properties will be more successful in correlating physico-chemical properties with biological activity.

CHAPTER IV NATURE OF PROTOHEMIN INHIBITION OF PORPHYRIN BIOSYNTHESIS

A. Introduction

Clarification of the steps of protoheme biosynthesis stimulated interest in the mechanism by which the rate of protoheme synthesis was controlled. Extensive studies employing bacterial systems have established that stimulation and inhibition of enzyme synthesis are important factors controlling biochemical pathways. In Bacillus subtilis a series of four enzymes is responsible for the degradation of L-histidine to ammonia, formamide, and L-glutamic acid. If L-histidine is omitted from the growth medium these enzymes show negligible activity. Addition of L-histidine to the medium results in a marked enhancement of the activity of these enzymes due to new enzyme synthesis (Chasin & Magasanik, 1968). Thus L-histidine acts as an inducer for the synthesis of enzymes responsible for its degradation.

In the bacterium Escherichia coli L-isoleucine, the end product of L-threonine metabolism, was shown to inhibit the synthesis of all the enzymes responsible for its formation from L-threonine. In addition L-isoleucine was shown to inhibit the activity of L-threonine deaminase, the first enzyme in the pathway. The former mechanism of control is described as repression and the latter negative feed-back or end-product inhibition (Changeux, 1965). Jacob & Monod (1961) proposed a model by which induction and repression of enzyme synthesis could be explained in bacteria. This model as applied by Granick (1966) to control of porphyrin biosynthesis has been explained in chapter I and shown diagrammatically in Fig. 7.

The work of several authors established that end-product inhibition is operative as a control mechanism in protoheme biosynthesis.

Lascelles (1956) demonstrated that Rhodopseudomonas spheroides, a photosynthetic bacterium, accumulates porphyrins in the medium when grown under iron-deficient conditions. A large decrease in porphyrin accumulation occurred on addition of small amounts of iron to suspensions of these organisms. Iron could not be acting solely by diverting porphyrins to iron porphyrins since the amount of iron added was only sufficient to convert 1% of the porphyrins to protoheme. This observation suggested that iron acted catalytically on porphyrin excretion by promoting the synthesis of the final product in the metabolic sequence, protoheme, which in turn inhibited the activity of the first enzyme in the sequence, δ -ALA synthetase. That this idea is probably correct has been demonstrated recently by the work of several groups. Thus Gibson et al. (1961) using crude cell extracts and Burnham & Lascelles (1963) using a partially purified δ -ALA synthetase preparation from R. spheroides have shown that protohemin inhibits the action of this enzyme. Burnham and Lascelles (1963) have shown that the inhibition by protohemin was reversible, thus providing additional support for the view that protohemin acts as a controlling factor since reversibility is a hallmark of end-product inhibition. Recently the effects of protohemin on the incorporation of glycine-2- ^{14}C and δ -ALA-4- ^{14}C into protoheme in immature rabbit erythrocytes has been examined (Karibian & London, 1965). The results of these studies indicated significant inhibition by protohemin of the utilization of glycine for protoheme synthesis; the effect on the utilization of δ -ALA was variable and less significant. These results have been interpreted as indicating that protoheme exerted a feedback control on its synthesis by inhibiting the activity of δ -ALA synthetase.

Evidence that repression may be important in the control of protoheme biosynthesis was first obtained by Lascelles (1960). Protohemin, when added to cultures of R. spheroides, inhibited the synthesis of δ -ALA synthetase. Granick (1966) has demonstrated in the chick embryo liver cell culture system that protohemin inhibited porphyria-induction by AIA (Fig. 5B). Since protohemin did not interfere with the enzymic conversion of δ -ALA to porphyrins nor alter the δ -ALA synthetase activity of guinea pig liver mitochondria Granick suggested that protohemin functions as a co-repressor for δ -ALA synthetase.

The idea that the inhibition by protohemin was specific for protoheme biosynthesis received support from the findings of Narisawa & Kikuchi (1966). These workers demonstrated in rat liver that AIA (Fig. 5B), besides inducing the formation of δ -ALA synthetase, induced the formation of the soluble enzymes δ -ALA dehydratase, glucose-6-phosphate dehydrogenase, and the microsomal enzyme NADP-cytochrome C reductase. The addition of protohemin to AIA treated rats inhibited only the formation of δ -ALA synthetase.

Following a single injection of protohemin into rats Waxman et al. (1966) reported cyclic variations in the activity of liver δ -ALA synthetase lasting for three days. These workers suggested that the initial excess of protohemin in the liver represses m-RNA synthesis resulting in decreased synthesis of δ -ALA synthetase and thus decreased activity of this enzyme. Subsequently protohemin is utilized for the formation of liver hemoproteins and bile pigments. This results in a lowered concentration of protoheme and new synthesis of the m-RNA for δ -ALA synthetase begins and consequently the activity of this enzyme

increases. This sequence of events brings about a succession of stop and go phases which cause oscillations in the level of δ -ALA synthetase.

Marver et al. (1966) showed that the administration of L-tryptophan to rats results in an increased activity of the protoheme enzyme tryptophan pyrrolase by increasing protoheme binding to the apoenzyme. In addition, new synthesis of δ -ALA synthetase was observed and it was suggested that increased protoheme binding by tryptophan pyrrolase reduces protoheme available for repression of δ -ALA synthetase. Consequently induction of δ -ALA synthetase takes place.

Narisawa and Kikuchi (1966) showed that following the administration of AIA to rats two phases in the induction of δ -ALA synthetase occurred. The first phase was noted within twelve hours after the first injection of AIA and a second more pronounced phase was observed after further injections of AIA, 12 and 24 hours later. While the first and second phases of induction were sensitive to inhibition by actinomycin D only the second phase was sensitive to inhibition by mitomycin C and 5-fluorouridine deoxyribose. According to modern theory actinomycin D inhibits DNA transcription to m-RNA and mitomycin C and 5-fluorouridine deoxyribose inhibit new DNA synthesis. These workers thus concluded that the first phase of δ -ALA synthetase induction results from increased DNA transcription to m-RNA and the second phase of induction requires new DNA synthesis (Fig. 25A).

Hayashi et al. (1968) repeated the experiment shown in Fig. 25A and showed that administration of actinomycin D to rats twenty-four hours after the first injection of AIA reduced the activity of δ -ALA synthetase when determined six hours later (Fig. 25B). The experiment was repeated

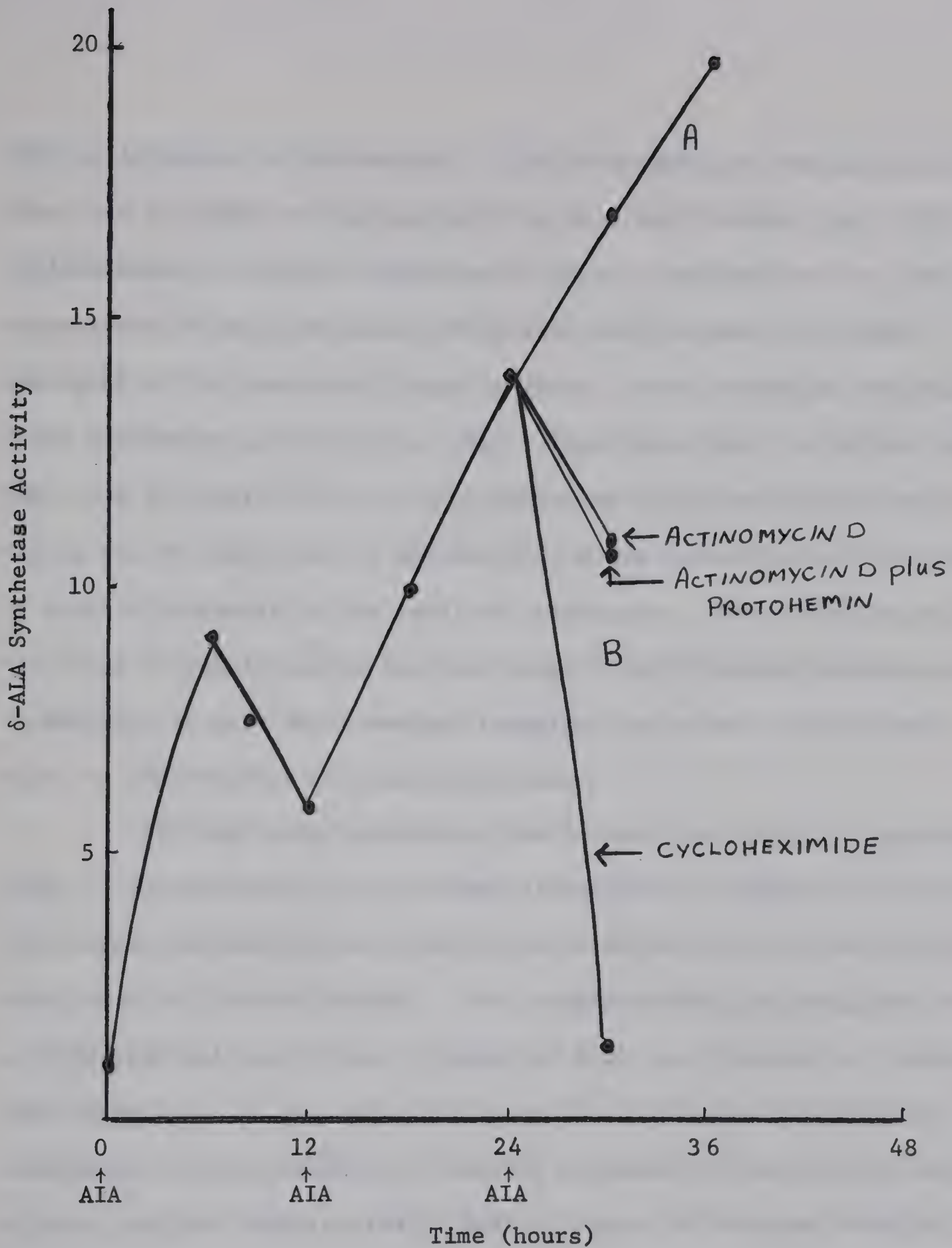


Fig. 25 A. The Two Phases of δ -ALA Synthetase Induction in Rat Liver Following Administration of AIA.

B. The Effect of Actinomycin D, Actinomycin D plus Protohemin, and Cycloheximide on the Induction of δ -ALA Synthetase (Narisawa & Kickuchi, 1966; Hayashi et al., 1968).

with an injection of actinomycin D plus protohemin and the effect produced was the same as that seen with actinomycin D alone (Fig. 25B). Cycloheximide, a specific inhibitor of protein synthesis at the level of translation (Young & Robinson, 1963) when administered in a manner analogous to the preceding drugs produced a more pronounced reduction in δ -ALA synthetase activity (Fig. 25B). From these data the authors suggest that the inhibition of δ -ALA synthetase induction by protohemin is mainly due to inhibition of synthesis of m-RNA rather than to inhibition of protein synthesis at the level of translation. Their conclusion is difficult to justify since only one dose of each drug was administered. In addition no data are presented revealing the effect of protohemin alone on the activity of δ -ALA synthetase.

All the above experiments lend support to Granick's hypothesis (Fig. 7) on the control of protoheme biosynthesis. They do not establish that porphyria-inducing drugs act at the same cellular site which is one requirement of Granick's model. That porphyria-inducing drugs may act at different cellular sites is suggested from the diversity of chemical structures known to induce porphyria and the fact that our preceding studies on the relationship of chemical structure to porphyria-inducing activity did not lead to clearly defined molecular features required for activity. We attempted to clarify this problem by studying the inhibitory effect of protohemin on a variety of drugs known to induce porphyria. If protohemin could be shown to inhibit some but not all of these drugs then Granick's model would require modification. These studies are reported in section B of this chapter.

Since Granick's model requires that protohemin repression be

competitive with respect to drug-induction we have studied the kinetic nature of protohemin inhibition of AIA-induced porphyria. These studies are reported in section C of this chapter.

Previous experiments have not clearly eliminated the possibility that protohemin may have additional sites, other than transcription, at which it may act to inhibit porphyria-induction. We have attempted in section D of this chapter to determine if protohemin inhibition of porphyria-induction is confined to the transcription level.

B. The Inhibitory Effect of Protohemin on Porphyria-Induction by a Variety of Compounds

Granick's model (Fig. 7) for drug-induced porphyria requires that all the drugs act at the same cellular receptor. As stated previously a wide variety of compounds with no obvious structural resemblance are potent porphyria-inducing drugs. Our studies of the relationship between structure and activity did not reveal an underlying common molecular feature among the drugs tested to which the property of porphyria-induction could be ascribed. For this reason it is difficult to visualize that these compounds all act at the same site to induce porphyria. Our next experiments were therefore designed to test the idea that all porphyria-inducing drugs act at a common site, viz., a site on the aporepressor normally occupied by protoheme. If protohemin could be shown to inhibit some but not all of these compounds this would clearly indicate that Granick's model requires modification and that porphyria can be induced by more than one mechanism. On the other hand, if protohemin inhibits porphyria-induction by a variety of potent

porphyria-inducing drugs, differing widely in chemical structure, this does not constitute proof of Granick's hypothesis. This follows from the fact that protohemin might act at a site distinct from that of the inducing drugs causing an inhibition of a step which is necessary for all porphyria-inducing drugs even though they may initially act at different loci. We have therefore studied the effect of protohemin on porphyria-induction by AIA (Fig. 5B), DDC (Fig. 5A), diethyl 2,3,5,6-tetramethylterephthalate (Fig. 12A), diethyl 3,3'-dimethylglutarate (Fig. 10G), griseofulvin (Fig. 8C), and glutethimide (Fig. 26).

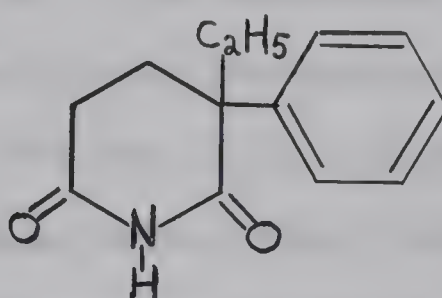


Fig. 26 Glutethimide

Experimental

i. Testing of Compounds for Porphyria-Inducing Activity in Cultures of Chick Embryo Liver Cells

Preparation of Reagents

Reagents were prepared as described in chapter II with the exception of the enzyme solution which was prepared as described below. This modified solution was used since it was shown that Pangestin was not required for the preparation of a cell suspension.

a. Preparation of Enzyme Solution for Digestion of Cells

Calcium and magnesium-free Earle's medium (5 ml) was

added to a solution of 2.5% trypsin in isotonic saline (4 ml, Microbiological Associates). This enzyme solution was prepared following the removal of the livers from the chick embryos and was always used immediately.

b. Preparation of Liver Cells

The method used was that described in chapter II, with the following modifications. After incubating the liver with enzyme solution at 37° for 15-30 minutes, the cell suspension was transferred to a conical centrifuge tube and spun at 2500 r.p.m. for 3 minutes. The supernatant was removed with a Pasteur pipette and the cells resuspended in 9 ml of fresh culture medium. Aliquots (0.2 ml) of this cell suspension were inoculated into glass petri dishes (Fisher Scientific, 5 cm internal diameter) containing 4 ml of culture medium. The cells were incubated at 37° in an atmosphere of moist 95% air, 5% CO₂ for 24 hours.

c. Counting of the Liver Cells

This has been described in chapter II. The average number of cells was 2.4×10^6 per 0.2 ml of suspension.

d. Addition of Chemicals to Liver Cells

The culture medium was removed from each petri dish after 24 hours of incubation and replaced with 4 ml of fresh medium. Chemicals were dissolved in 95% re-distilled ethanol (5 λ) and added to the liver cells by means of disposable lambda pipettes. After the addition of chemicals plus protohemin the petri dishes were returned to the incubator for approximately twenty-four

hours.

c. Properties of Protohemin

The protohemin used in these studies was prepared previously according to the method described by Shemin (1957). According to Falk (1964) the best procedure for determining the purity of protohemin is to determine its hemochrome spectrum. For this reason this spectrum was determined as described by Falk (1964).

Protohemin (0.17 mg) was dissolved in redistilled pyridine (1.7 ml) and 0.5 ml of this solution was added to a glass cuvette of 1 cm path length containing 1 ml of 0.25 N NaOH and 1.5 ml distilled water to make a final volume of 3 ml. A few crystals of sodium dithionite were added to the cuvette and the visible spectrum of the pyridine hemochrome was run as quickly as possible employing a Bausch and Lomb spectronic 505 spectrophotometer. A few more crystals of sodium dithionite were added until no further increase in absorbency was noted. The second cuvette contained 0.5 ml pyridine, 1 ml 0.25 N NaOH and 1.5 ml distilled water and was used as a blank. The spectrum is shown in Fig. 27.

The α and β absorption peaks and the trough between these peaks are located at 556.8, 525, and 540 m μ . Falk (1964) records values of 557, 526, and 540 m μ for the corresponding peaks and troughs. The ϵ value for the α peak was found to be $31 \text{ (cm)}^{-1} \text{ (l)} \text{ (mM)}^{-1}$ and the ratio between the maximum of the α peak and the minimum between the α and β peaks was 3.26. Falk (1964) records an ϵ value of $34.4 \text{ (cm)}^{-1} \text{ (l)} \text{ (mM)}^{-1}$ for the α peak and records a value of 3.46 for this ratio.

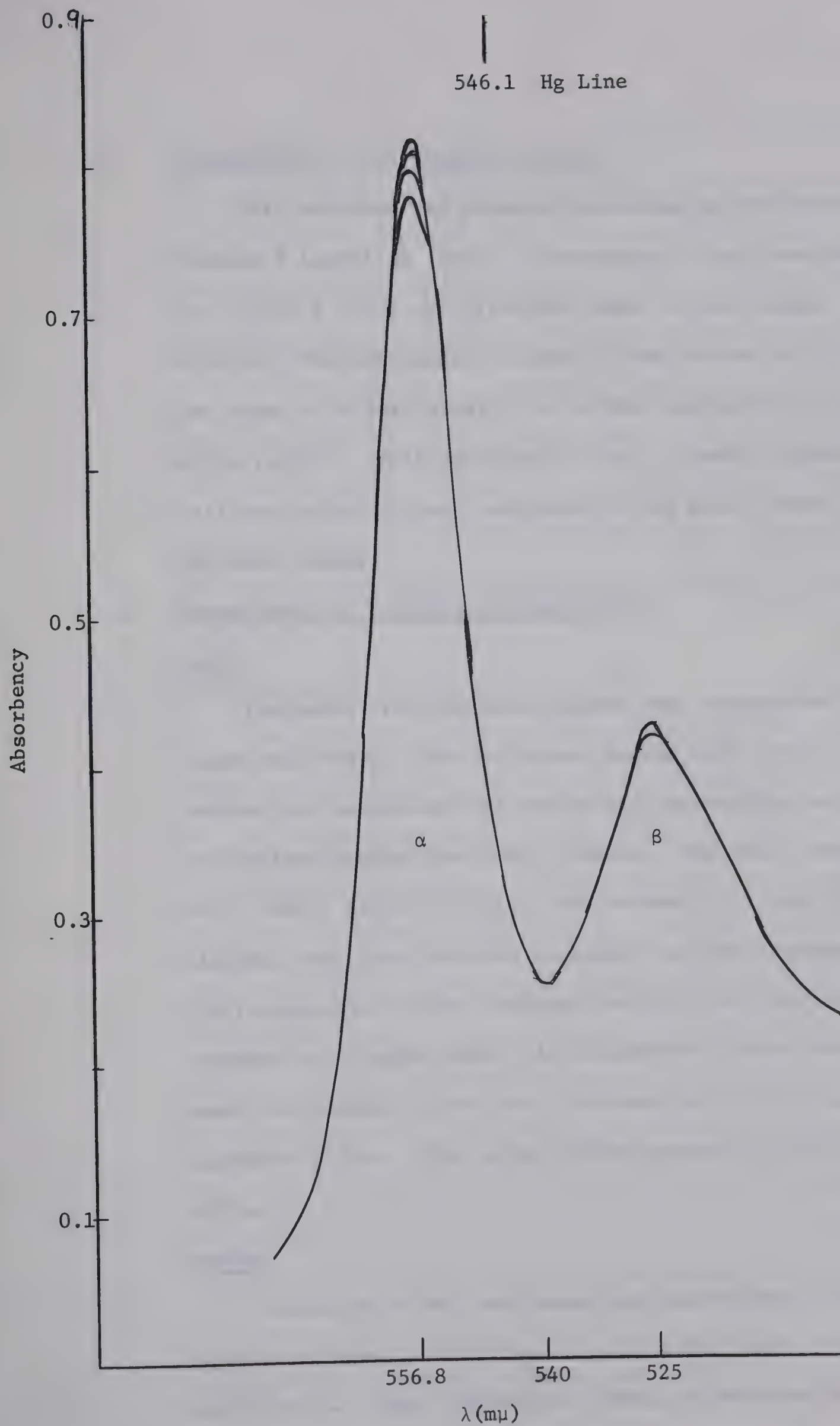


Fig. 27 Pyridine Hemochrome Spectrum of Protoheme

f. Preparation of Protohemin Solution

This solution was prepared according to the method of Burnham & Lascelles (1963). Protohemin (15 mg) was dissolved in 1 ml of 1 N KOH and distilled water (10 ml) added. 9 ml of 0.01 N HCl were added to make a final volume of 20 ml. A few drops of dilute alkali (0.1 N KOH) were added to adjust the pH to 7.8-8.0. This solution (0.1 ml), freshly prepared one-half hour prior to use, was added to the petri dishes containing the liver cells.

g. Measurement of Porphyrin Accumulation

Cells

The media from the petri dishes were transferred to test tubes and frozen. The cells were washed with 2 ml of Earle's calcium and magnesium-free medium and the washing was added to the culture medium previously removed. The cells were treated at 4° with 4 ml of 1 M HClO_4 : 98% ethanol (1:1 v/v) for 5 minutes, the clear solution decanted, and the treatment repeated. The fluorescence of the combined porphyrin extract was then measured in a Turner model 110 fluorometer fitted with a 405 mμ band pass primary filter and a Wratten No. 25 (595 mμ) sharp secondary filter. The latter filter passes all light above 595 mμ.

Medium

The medium (6 ml) was thawed and poured into a 60 ml separatory funnel containing 10 ml of ether and 0.2 ml glacial acetic acid. After shaking the funnel an emulsion formed at the

boundary of the two phases. The major part of the aqueous phase was discarded and the remaining mixture containing the ether phase and the emulsion was transferred to centrifuge tubes and centrifuged at 2500 r.p.m. for 4 minutes. The ether layer containing the porphyrins was transferred by means of a Pasteur pipette to a separatory funnel and the porphyrins extracted into 2 ml of 1 M perchloric acid. The perchloric acid was transferred to test tubes containing 2 ml of 98% ethanol and the fluorescence measured. The total porphyrin accumulation is the sum of the porphyrin content of cells and medium.

h. Calibration of the Fluorescence Curve

Granick (1966) has shown that the porphyrins produced by induced cells consisted of over 80% coproporphyrin III with di- and tricarboxylic acid porphyrins as the remainder. For this reason a fluorescence calibration curve was constructed with coproporphyrin I as a standard. This porphyrin has the same fluorescence properties as coproporphyrin III and is more readily available. The procedure used was that described by Talman (1958) as modified by Schwartz. The tetramethyl ester of coproporphyrin I (0.535 mg) was transferred quantitatively to a volumetric flask (100 ml) to which was added 1 ml of 7.5 N HCl and the ester was allowed to hydrolyze overnight. The flask was filled to the 100 ml mark with a solution of 1 M HClO_4 : 98% ethanol (1:1 v/v). The concentration of coproporphyrin I in this solution was 493 $\mu\text{g}/100\text{ ml}$.

Appropriate dilutions of this solution were made and the fluorescence measured. The calibration curve is shown in Fig. 28.

i. Determination of the Protein Content of the Cells

The method used was a modification by Miller (1959) of the colorimetric determination of protein by Lowry et al. (1951). A standard curve was prepared employing bovine serum albumin as the protein source in concentrations of 50 to 200 $\mu\text{g/ml}$ of distilled water. Reagents used were the following:

1. Folin-Phenol Reagent

Folin-Phenol reagent (5 ml; BDH) diluted with 50 ml of distilled water.

2. Copper Reagent

1 ml 1% copper sulfate

1 ml 2% sodium/potassium tartrate

20 ml 10% sodium carbonate in 0.5 N NaOH

These reagents were freshly prepared from stock solutions one-half hour prior to use. To 1 ml of the protein solution was added 1 ml of the copper reagent and the resulting solution was allowed to stand 10 minutes at room temperature. Phenol reagent (3 ml) was added and the solution placed in a water bath at 50° for 10 minutes whereupon a blue colored complex developed. The absorption of this complex was measured at 540 $\text{m}\mu$. The

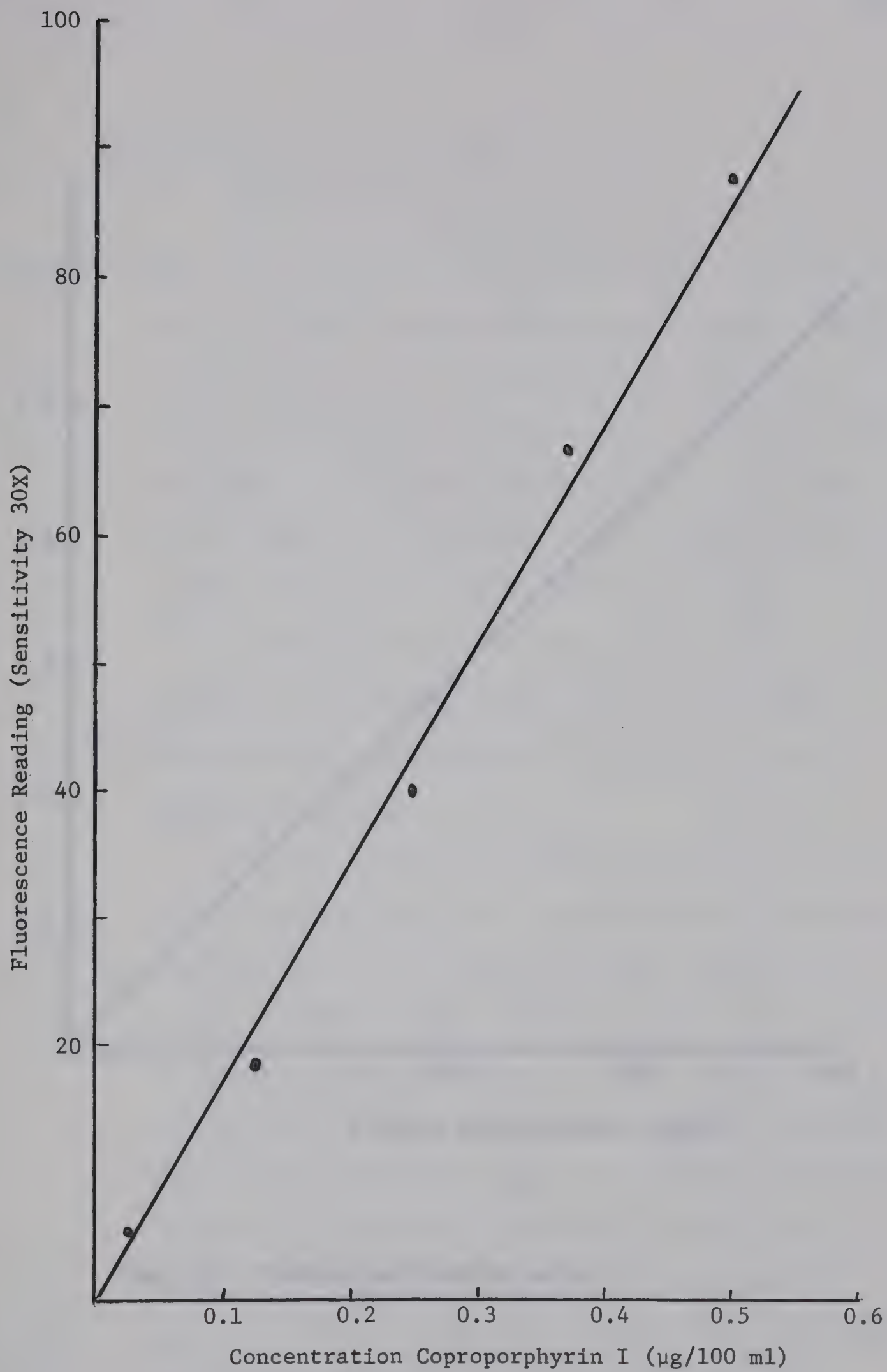


Fig. 28 Fluorescence Calibration Curve

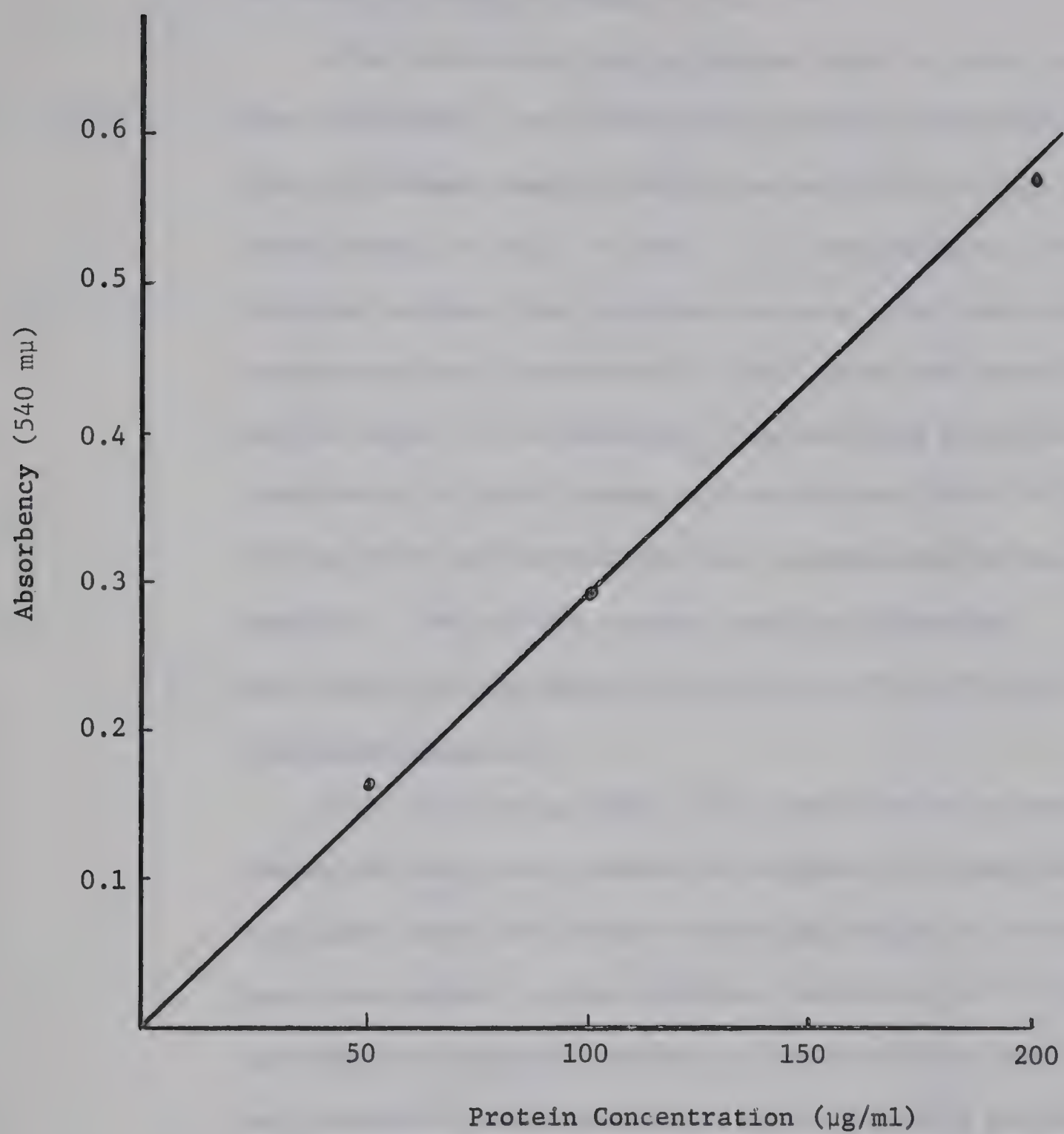


Fig. 29 Protein Calibration Curve

calibration curve is shown in Fig. 29.

After extracting the porphyrins from the cells as described above, the residues were rinsed consecutively with 98% ethanol and 98% ethanol-ether solution (3:1 v/v). After drying in air, 1 N NaOH (4 ml) was added and the residues scraped from the glass surface. The resulting suspensions were transferred to test tubes and immersed in boiling water for 10 minutes. The resulting solutions were transferred to petri dishes and neutralized with 1 N HCl. If this step was omitted the blue colored complex was unstable. The protein content was then determined.

ii. Measurement of the Inhibitory Effect of Protohemin on Porphyrin-Induction

After incubating liver cells for 24 hours in petri dishes the media were removed and replaced by fresh media. Two hours later the porphyria-inducing drugs and protohemin were added. After a further incubation of 24 hours duration the porphyrin content of both cells and media was determined and expressed as μg porphyrin/mg protein. The results of these experiments are shown in table XIV. Each drug was tested at a particular concentration in duplicate or triplicate. In addition the amount of porphyrin in cultures to which ethanol (5 λ) alone was added is recorded.

TABLE XIV THE INHIBITORY EFFECT OF PROTOHEMIN ON A VARIETY OF POTENT
PORPHYRIA-INDUCING DRUGS

Porphyria-Inducing Drug	Concentration ($\mu\text{m}/\text{ml}$)	Protohemin Concentration ($\mu\text{m}/\text{ml}$)	Porphyrin Accumulation	
			($\mu\text{g}/\text{mg}$ Protein)	Mean
AIA (Fig. 5B)	4.3		0.529 0.533	0.531
	4.3	0.024	0.135 0.111	0.123
	2.1		0.618 0.544	0.581
	2.1	0.024	0.055 0.068	0.062
	0.028		0.096 0.090	0.093
	0.028	0.024	0.027 0.022	0.025
DDC (Fig. 5A)	0.036		0.161 0.195 0.215	0.190
	0.036	0.021	0.072 0.083 0.071	0.075
	0.018		0.076 0.081 0.056	0.071
	0.018	0.021	0.032 0.025 0.040	0.032
	0.009		0.050 0.027 0.029	0.035
	0.009	0.021	0.013 0.012 0.010	0.012
Griseofulvin (Fig. 8C)	0.057		0.485 0.464	0.475
	0.057	0.026	0.024 0.020	0.022
	0.029		0.066 0.078	0.072
	0.029	0.026	0.019 0.015	0.017
	0.006		0.024 0.021	0.023
	0.006	0.026	0.009 0.008	0.009

TABLE XIV (continued). THE INHIBITORY EFFECT OF PROTOHEMIN ON A VARIETY OF POTENT PORPHYRIA-INDUCING DRUGS.

Porphyria-Inducing Drugs	Concentration ($\mu\text{m}/\text{ml}$)	Protohemin Concentration ($\mu\text{m}/\text{ml}$)	Porphyrin Accumulation	
			($\mu\text{g}/\text{mg}$ Protein)	Mean
Diethyl 3,3'- dimethylglutarate (Fig. 10G)	0.93		0.649 0.878	0.764
	0.93	0.026	0.318 0.273	0.296
	0.19		0.021	0.021
	0.19	0.026	0.013	0.013
Diethyl 2,3,5,6- tetramethyl- terephthalate (Fig. 12A)	0.25		1.054 0.871	0.963
	0.25	0.026	0.221 0.190	0.205
	0.025		0.572 0.445	0.509
	0.025	0.026	0.075 0.080	0.078
	0.014		0.017 0.022	0.020
	0.014	0.026	0.013 0.012	0.013
Glutethimide (Fig. 26)	0.46		0.946 1.134	1.040
	0.46	0.026	0.176 0.107	0.142
	0.046		0.756 0.207	0.482
	0.046	0.026	0.029 0.029	0.029
	0.009		0.017 0.021	0.019
	0.009	0.026	0.010 0.010	0.010
CONTROLS			0.008 0.008 0.008 0.011 0.008	0.009

Discussion

The results shown in table XIV clearly reveal that protohemin inhibits the action of porphyria-inducing drugs of diverse chemical structure. This finding is consistent with Granick's model (Fig. 7). However, the results are also consistent with the possibility that protohemin inhibits some other step common to all drugs that induce porphyria, e.g., inhibition of δ -ALA synthetase. The mechanism of action of protohemin will be considered further after taking into consideration the results in chapter IV C and IV D.

C. The Kinetic Nature of Protohemin Inhibition of Porphyria-Induction

Granick's model requires that the porphyria-inducing drugs and protohemin compete for the same site on an aporepressor. Molecules that have an affinity for the same site would be expected to have close structural similarity. As discussed previously a wide variety of chemical structures can induce porphyria. It is therefore difficult to visualize all of these compounds acting at the same site. Furthermore it is even more difficult to visualize a structural similarity between any porphyria-inducing drug and protohemin which would allow both compounds to act at the same site. If protohemin and porphyria-inducing drugs act at the same site then protohemin inhibition would be competitive. If, however, protohemin acts at a site different from the porphyria-inducing drugs then protohemin inhibition should be noncompetitive. In addition we could have the special case of protohemin acting at a site on the aporepressor sufficiently close to that of the porphyria-inducing drug for competitive kinetics to be observed. In our next experiments we therefore studied the kinetic nature

of the inhibition by protohemin of porphyria-induction by AIA.

Fig. 30A illustrates the concept of competitive inhibition (Ariëns, 1964). The drug A is in reversible equilibrium with a site on the receptor R and this interaction leads to an effect E. Drug B is also in reversible equilibrium with a site on the receptor R but such interaction does not lead to an effect. The presence of drug B results in a parallel shift of the log-dose response curves for drug A to higher concentrations. The competition between A and B is shown by the fact that the influence of B can be overcome by an increase of the dose of A, i.e., the antagonism is surmountable. In Granick's model A would be a porphyria-inducing drug, B protohemin, R the aporepressor, and E the accumulation of porphyrin in chick embryo liver cells.

Fig. 30B illustrates the concept of noncompetitive inhibition (Ariëns, 1964). According to this model the porphyria-inducing drug A reacts with the aporepressor R leading to accumulation of porphyrin E. Protohemin reacts with a receptor R' distinct from the aporepressor such that the effect of A is inhibited. In this case the maximum porphyrin accumulation is determined only by the concentration of protohemin. There is no shift in the curves but a gradual decline in the maximum and a disappearance of porphyrin accumulation at high concentrations of protohemin. The antagonistic action of a sufficiently high dose of protohemin is insurmountable by the highest doses of the porphyria-inducing drug. If lower doses of both the porphyria-inducing drug and protohemin are used the decrease in the effect caused by protohemin may be reduced by an increase of the dose of the porphyria-inducing drug. In this case the

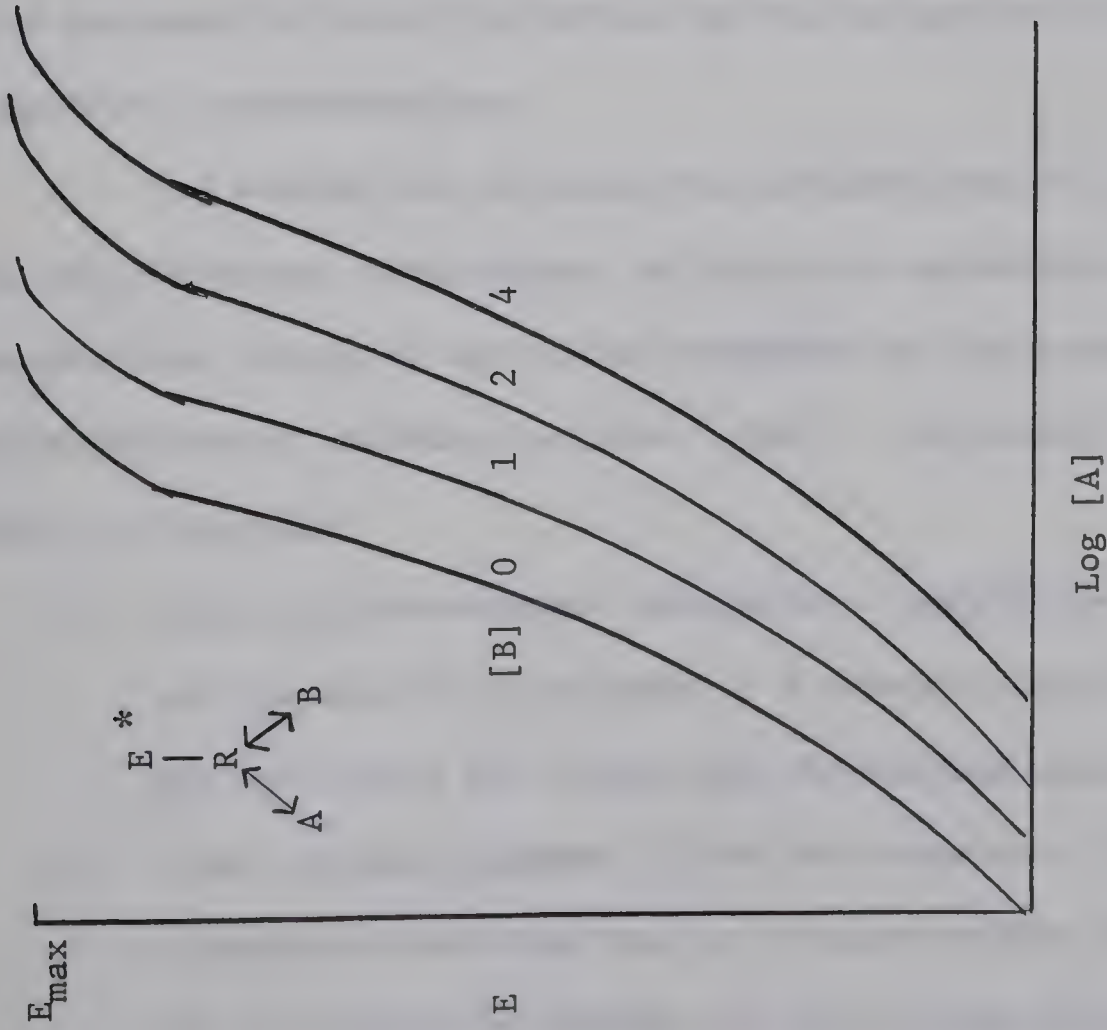


Fig. 30A Theoretical Log Concentration-Response Curves for an Agonistic Compound A with Various Concentrations of a Competitive Antagonist B (Ariëns, 1964).

* E = Biological Effect
 R = Receptor
 A = Agonist
 B = Competitive Antagonist

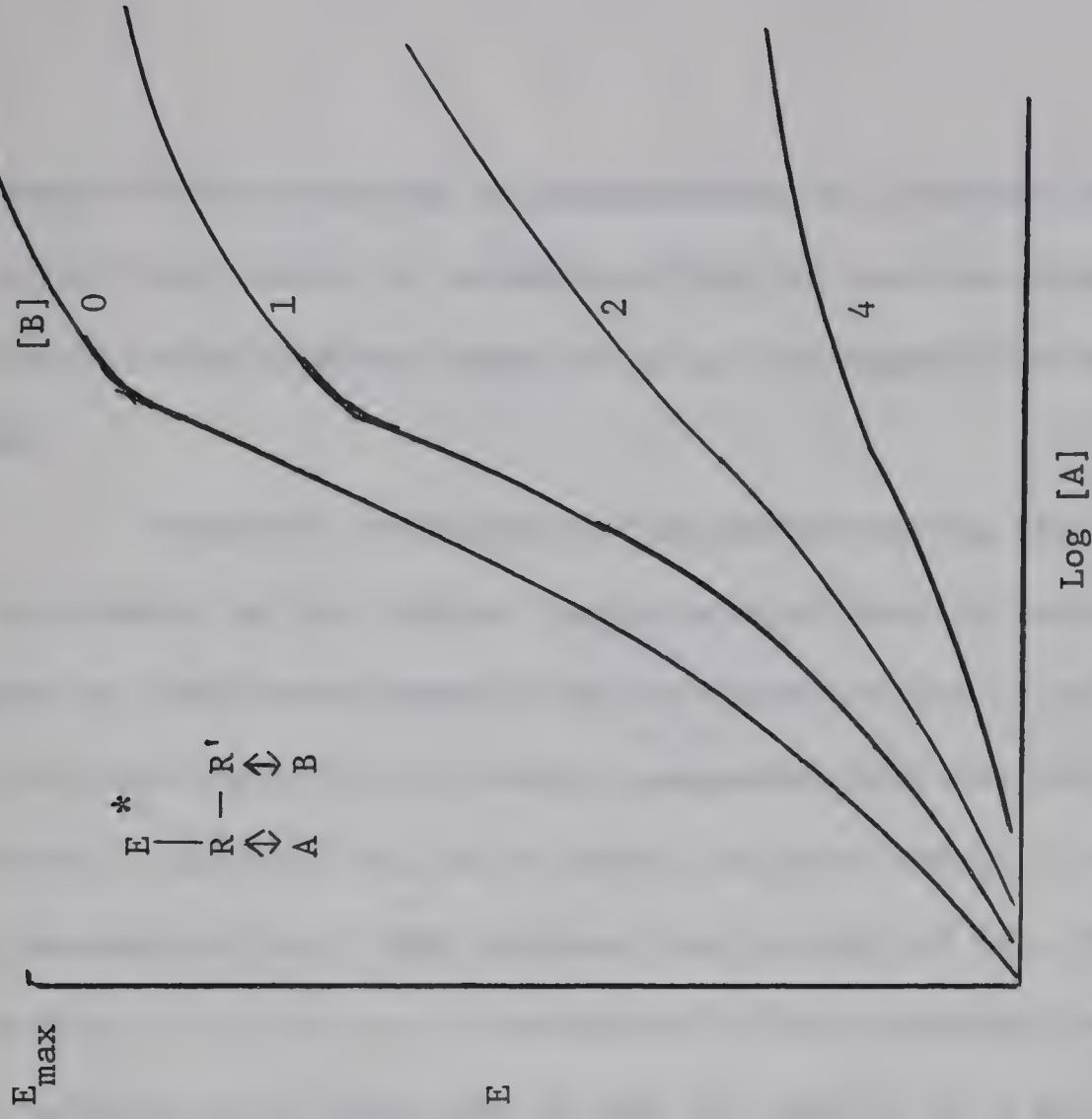


Fig. 30B Theoretical Log Concentration-Response Curves for an Agonist A Combined with Various Concentrations of the Noncompetitive Antagonist B. (Ariëns, 1964)

* E = Biological Effect
 R = Receptor
 R' = A Receptor Distinct from R
 A = Agonist
 B = Noncompetitive Antagonist

noncompetitive antagonism is surmountable to a certain degree. This must be taken into account if surmountability is used as an argument in the differentiation between competitive and noncompetitive antagonism (Ariëns, 1964).

Granick's experimental data demonstrating the inhibitory effect of protohemin on AIA induced porphyria are shown in table XV. Since the degree of inhibition depended on the concentration of protohemin relative to the fixed dose of AIA, Granick suggested that the inhibition was competitive. However, one would expect the same result if the inhibition was noncompetitive. This follows from a study of Fig. 30 A,B. It can be seen that in either case a reduction in the concentration of protohemin (B) relative to a fixed dose of AIA (A) results in a greater effect E. Thus in order to differentiate competitive from noncompetitive inhibition it is necessary to study the effect of the antagonist B over a full range of agonist A concentrations.

The easiest way to study the interactions of a porphyria-inducing drug and protohemin with respect to porphyrin accumulation is to construct dose-response curves of one of the compounds in the presence of constant concentrations of the other (Ariëns, 1964). This means that two sets of curves are possible:

1. A set of dose-response curves for a porphyria-inducing drug in the presence of protohemin in a concentration which is constant for each curve but varies for the various curves.
2. A set of dose-response curves for protohemin in the presence of a porphyria-inducing drug in a concentration which is constant for each curve but varies for the various curves.

TABLE XV INHIBITORY EFFECT OF PROTOHEMIN ON PORPHYRIN SYNTHESIS INDUCED BY AIA IN CULTURES OF CHICK EMBRYO LIVER CELLS (COVER SLIP TECHNIQUE WAS EMPLOYED FOR CULTURE OF CELLS; Granick, 1966).

Compound	Protohemin Concentration ($\mu\text{g/ml}$)	Coproporphyrin ($\times 10^{-11}$ moles/mg Protein) (mean plus average deviation of triplicate analysis)
Control minus AIA		1.5 ± 0.1
Control with AIA		14.0 ± 2.0
AIA (15 $\mu\text{g/ml}$) with Protohemin	5.0	1.4 ± 0.1
	1.5	6.1 ± 2.3
	0.5	10.1 ± 4.6

In the following experiments we have obtained a set of dose-response curves for AIA in the presence of protohemin in a concentration which is constant for each curve but varies for the various curves. Ideally it would have been desirable to construct a complete set of curves in one experiment. However, from a practical point of view this was not possible. For this reason in most experiments the log-dose response curve for AIA in the absence and presence of one given dose of protohemin was obtained.

Experimental

Liver cells from 17 day chick embryos were grown in primary cell culture as described in chapter IV, e. After incubation for 24 hours the medium was removed and replaced with 4 ml of fresh medium. Two hours later AIA and protohemin were added. Following a further incubation of 24 hours porphyrin accumulation in cells and medium was determined. After determining the protein content of the cells porphyrin accumulation was expressed as μg porphyrin per mg protein and log-dose response curves were constructed. Each point on the curves represents the mean of triplicate analysis and the range is also shown.

Results and Discussion

In Fig. 31 is shown a set of dose-response curves for AIA in the presence and absence of various concentrations of protohemin. The following features of this set of curves were of interest: (1) The dose-response curves for AIA in the presence of increasing concentrations of protohemin were shifted progressively to the right but in non-parallel fashion.

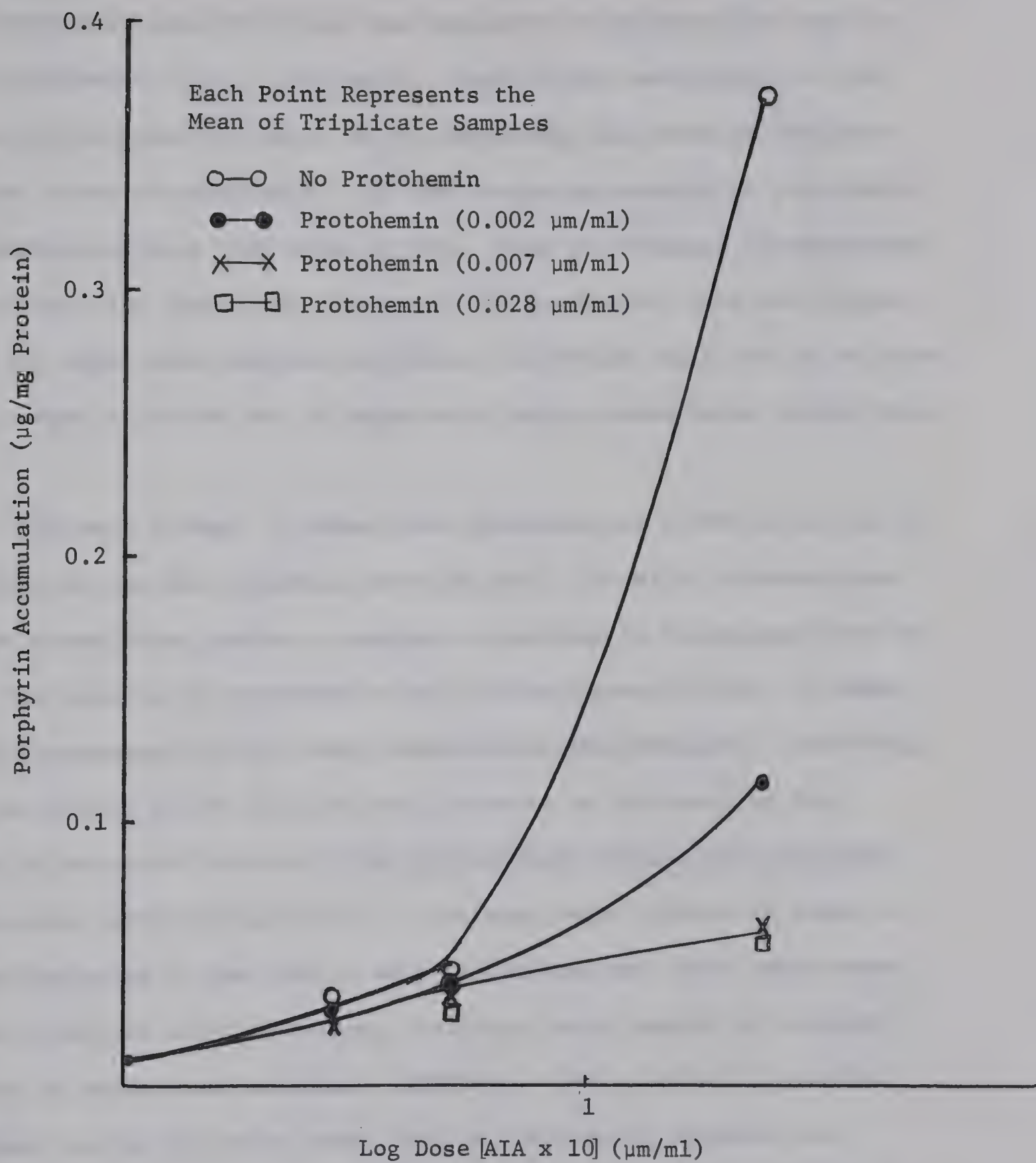


Fig. 31 Dose Response Curve for AIA in the Presence of Various Concentrations of Protohemin

(2) The porphyria-induction by AIA was sensitive to inhibition by very low doses of protohemin, viz., $0.002 \mu\text{m}/\text{ml}$. Despite this sensitivity to low doses, porphyria-induction would not be completely abolished by considerably larger doses of protohemin. (3) The antagonism exerted by protohemin was non-surmountable at high doses of AIA. Thus the evidence is consistent with noncompetitive inhibition. However, the possibility that even higher doses of AIA might have overcome protohemin inhibition could not be excluded. For this reason a further set of experiments were planned using higher doses of AIA.

The data in Fig. 32 reveal that protohemin at $0.0002 \mu\text{m}/\text{ml}$ has a small effect on the dose-response curve for AIA. It was of interest that porphyrin accumulation reaches a maximum in response to increasing doses of AIA and then apparently decreases at still higher doses of AIA. To understand this phenomenon the following observations are pertinent: According to Granick healthy growth of liver cell colonies is indicated by the presence in peripheral cells of filmy protoplasmic projections spreading over the glass surface (Fig. 11A). In contrast toxic effects of drugs on cells are indicated by the loss of such projections and their replacement by smooth spherical cell boundaries. Cellular death results in complete detachment of cells from the glass. Examination of cells which received the highest dose of AIA under phase contrast microscopy revealed the presence of filmy protoplasmic projections in peripheral cells similar to that seen in cells which received no AIA and those which received the smaller doses of AIA. These observations suggested that the apparent inhibition of porphyrin accumulation by high doses of AIA was not due to toxicity and might be relevant to control of protoheme biosynthesis.

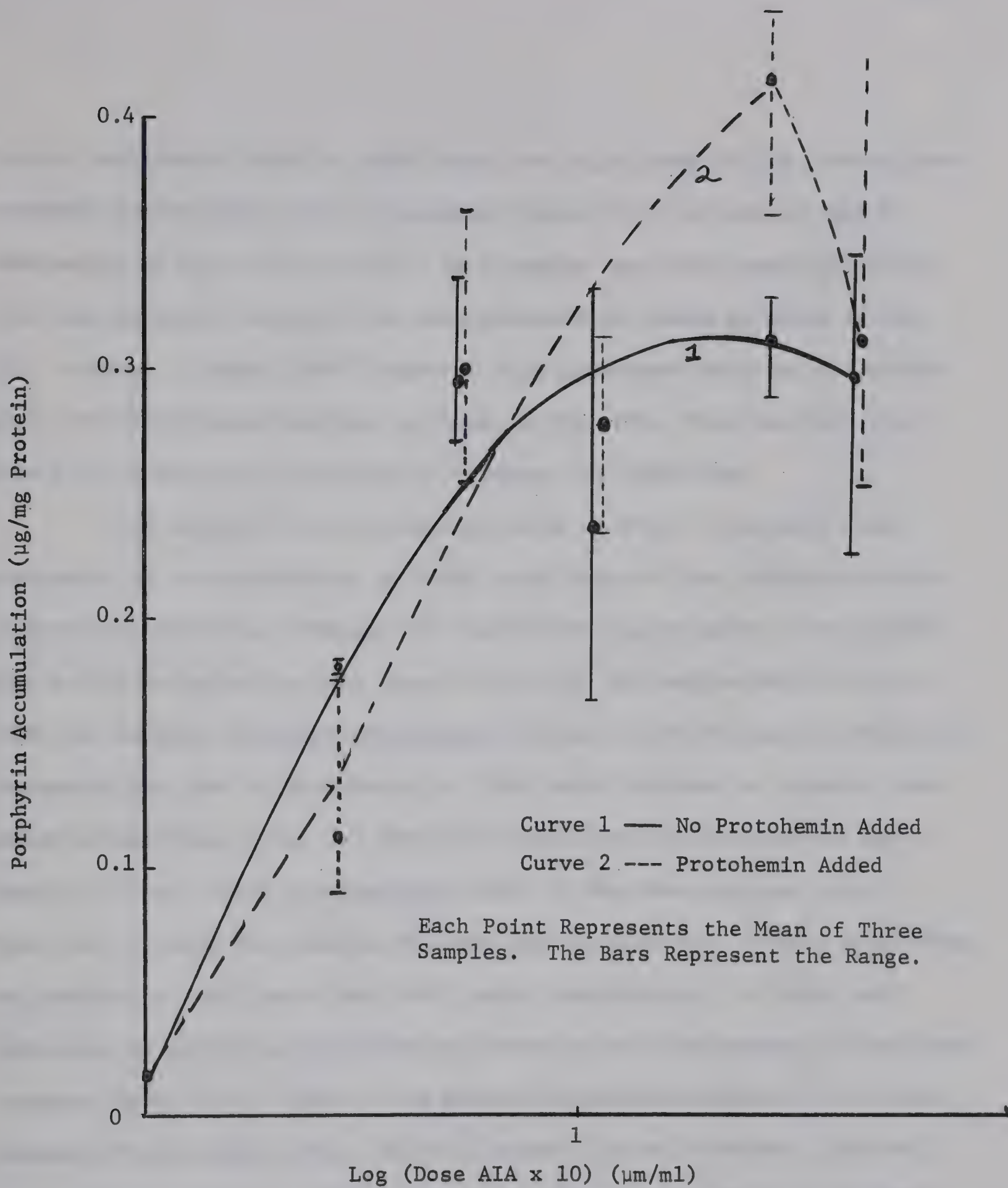


Fig. 32 Dose Response Curves for AIA in the Presence and Absence of Protohemin ($0.0002 \mu\text{m/ml}$)

Further experiments clearly established that high doses of AIA produce less porphyrin accumulation than intermediate doses of AIA as can be seen by examination of Figs. 34, 36 & 37. In a similar way high doses of DDC produce less porphyrin accumulation than intermediate doses as shown in Fig. 33A. Granick & Kappas (1967) observed this phenomenon with the porphyrinogenic steroid etiocholanolone as shown in Fig. 33B. They ascribed this effect to toxicity but presented no evidence for this idea.

The results of the experiment shown in Fig. 34 indicate that protohemin at a concentration of $0.002 \mu\text{m/ml}$ has a slight inhibitory effect on porphyria-induction although the results are inconclusive. The inhibition is not as marked as that shown in Fig. 31, and emphasizes the need to carry out several different experiments because of the biological variation. Increasing the dose of protohemin to $0.008 \mu\text{m/ml}$ produces an apparent competitive inhibition (Fig. 35) since the inhibition was overcome by high doses of AIA and there is a parallel shift in the dose-response curve. Figs. 36 & 37 show the results obtained from experiments in which protohemin was present at $0.005 \mu\text{m/ml}$ and $0.01 \mu\text{m/ml}$ respectively. In each case inhibition of porphyria-induction was shown by the displacement of the dose-response curves to the right. The shape of the dose-response curve in the presence of protohemin (Figs. 36 & 37, curve 2) is of interest. Thus at very high doses of AIA in the presence of a fixed concentration of protohemin there is less porphyrin accumulation than that observed for moderate doses of AIA. This finding is inconsistent with the simple model proposed by Granick (Fig. 7). From the slopes of the curves it is difficult to decide whether the displacement of the curves is parallel or non-parallel. In Fig. 38 the effect of $0.015 \mu\text{m/ml}$ protohemin is shown. There is marked

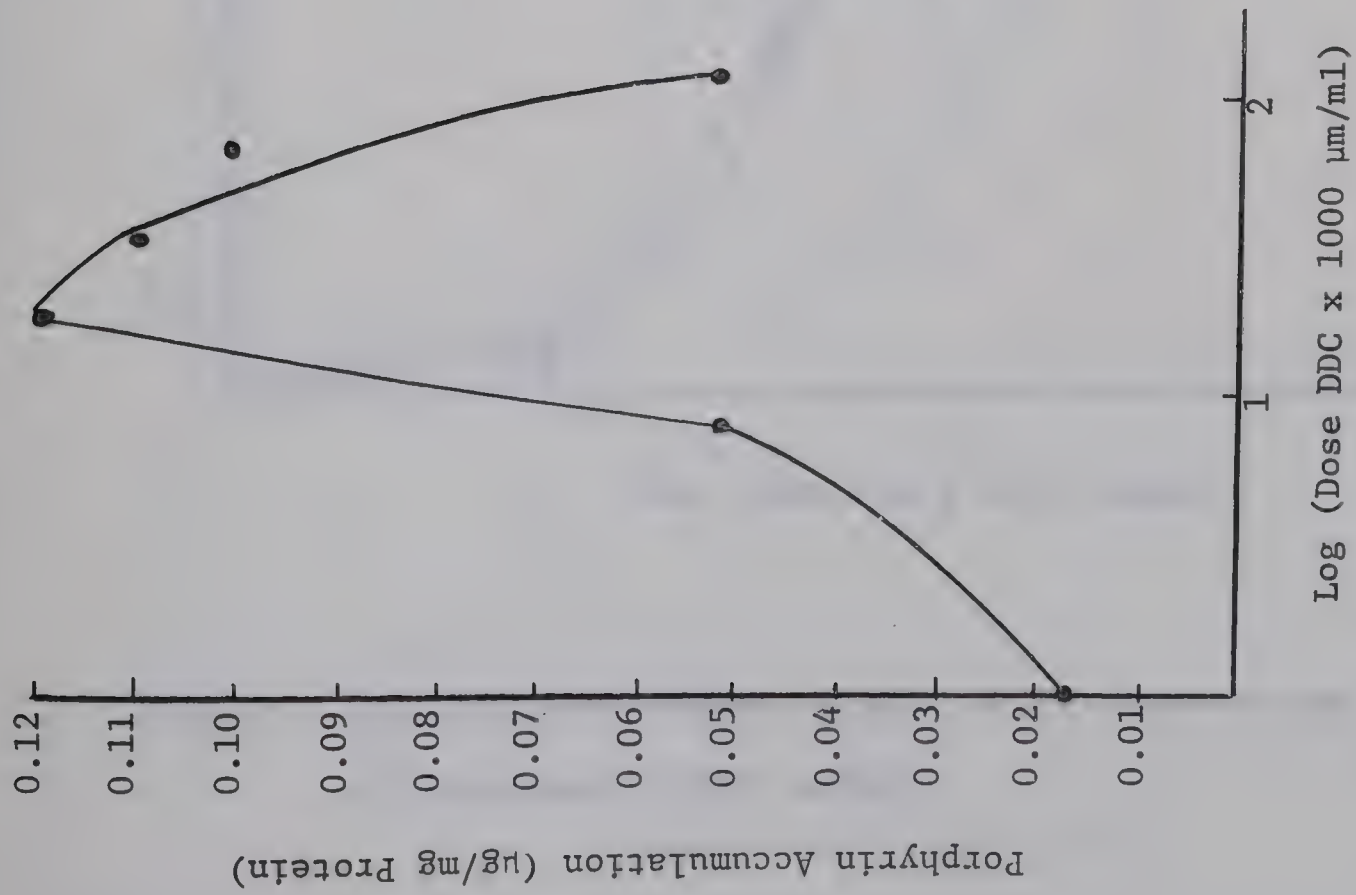


Fig. 33A Dose Response Curve for DDC.

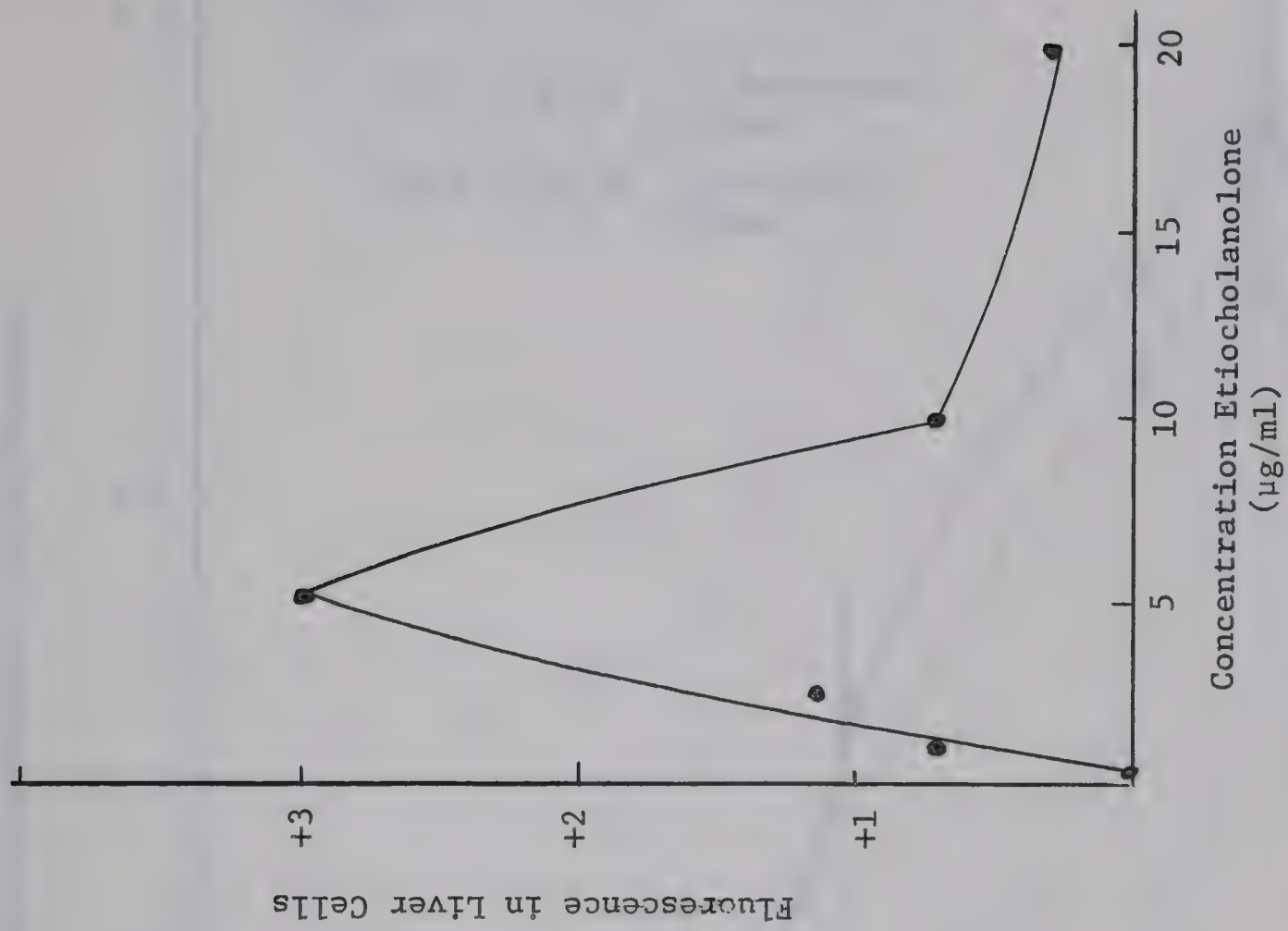


Fig. 33B Dose Response Curve for Etiocholanolone.
(Granick & Kappas, 1967)

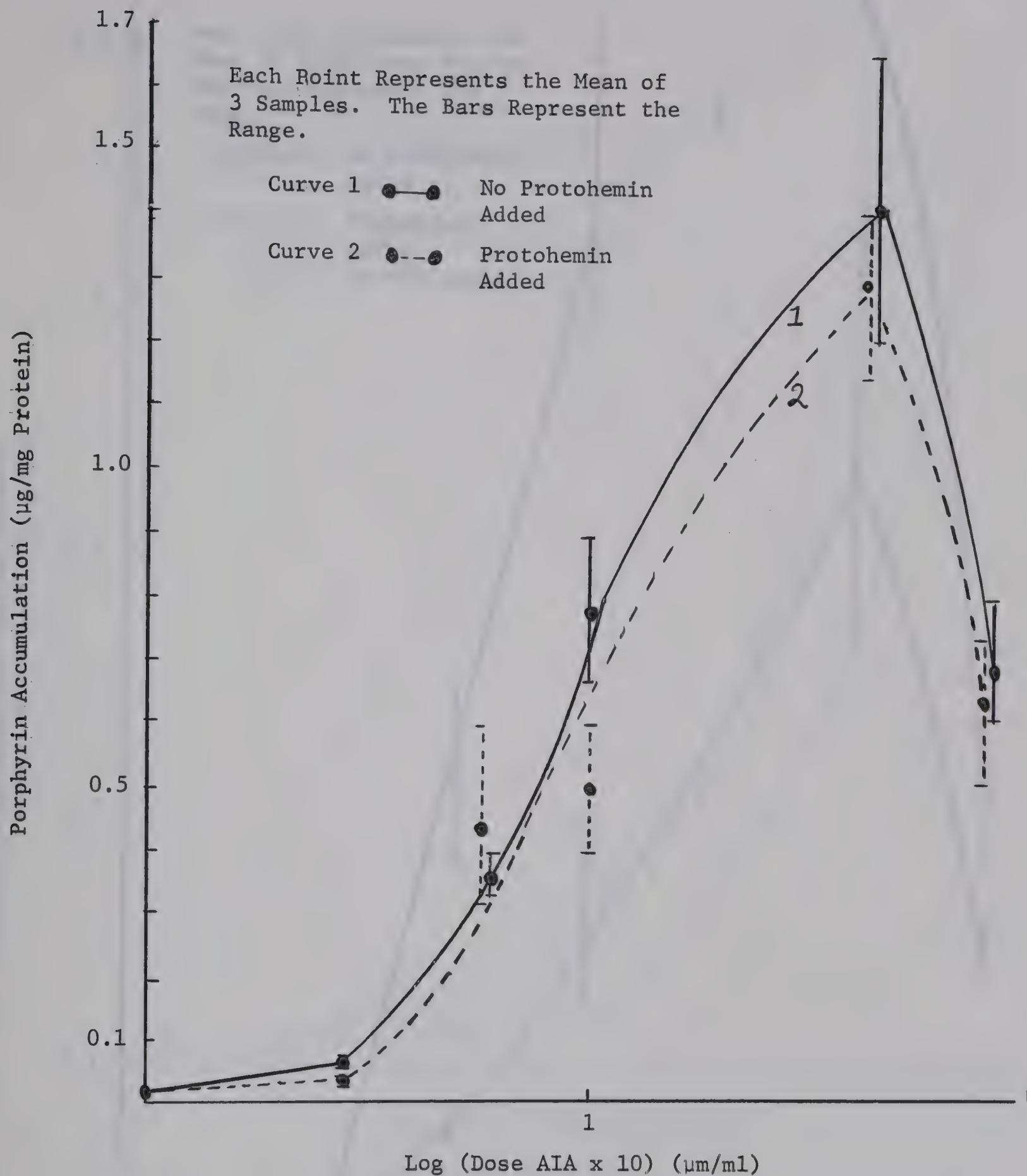


Fig. 34 Dose Response Curves for AIA in the Presence and Absence of Protohemin ($0.002 \mu\text{m}/\text{ml}$).

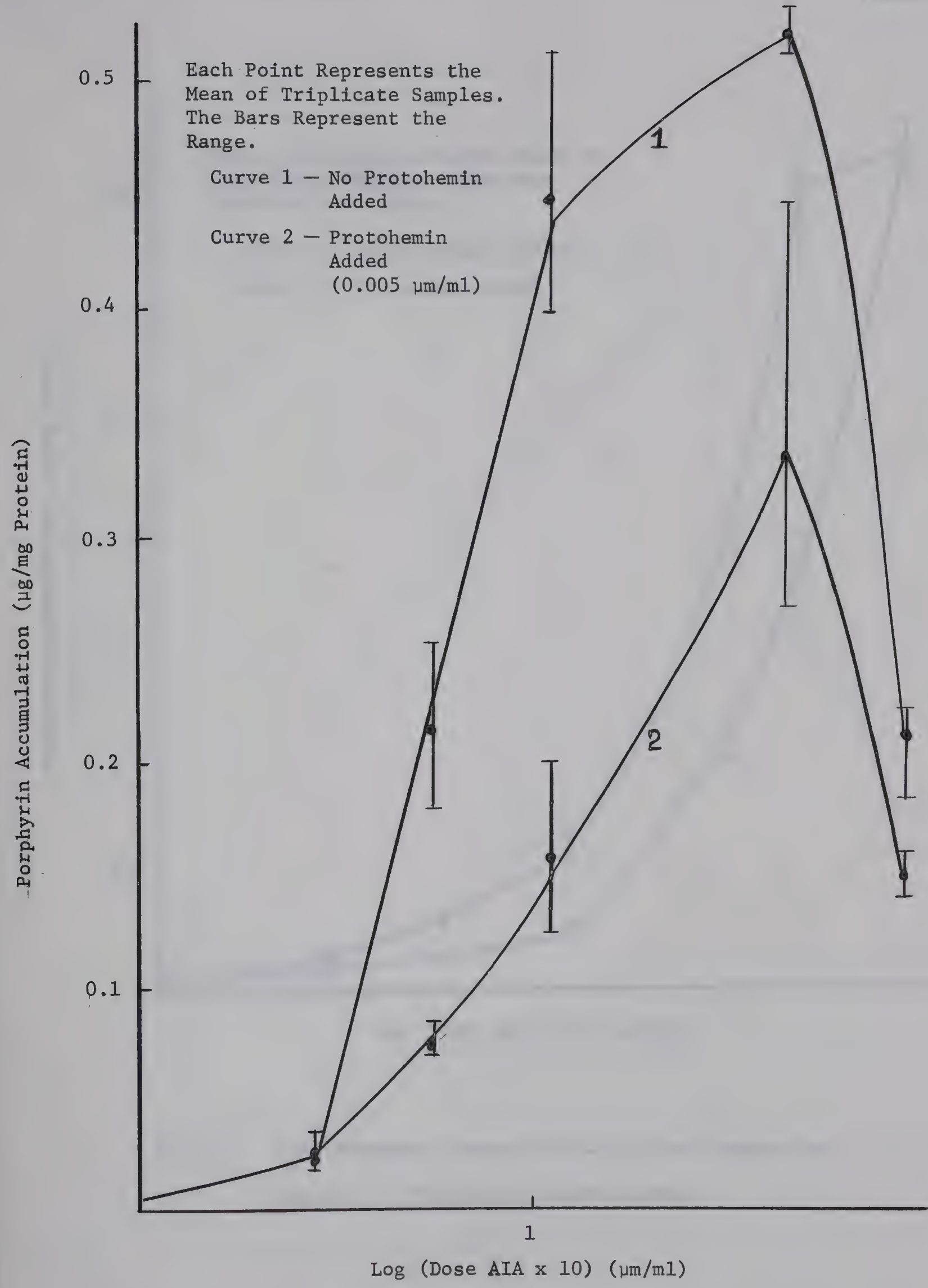


Fig. 36 Dose Response Curves for AIA in the Presence and Absence of Protohemin.

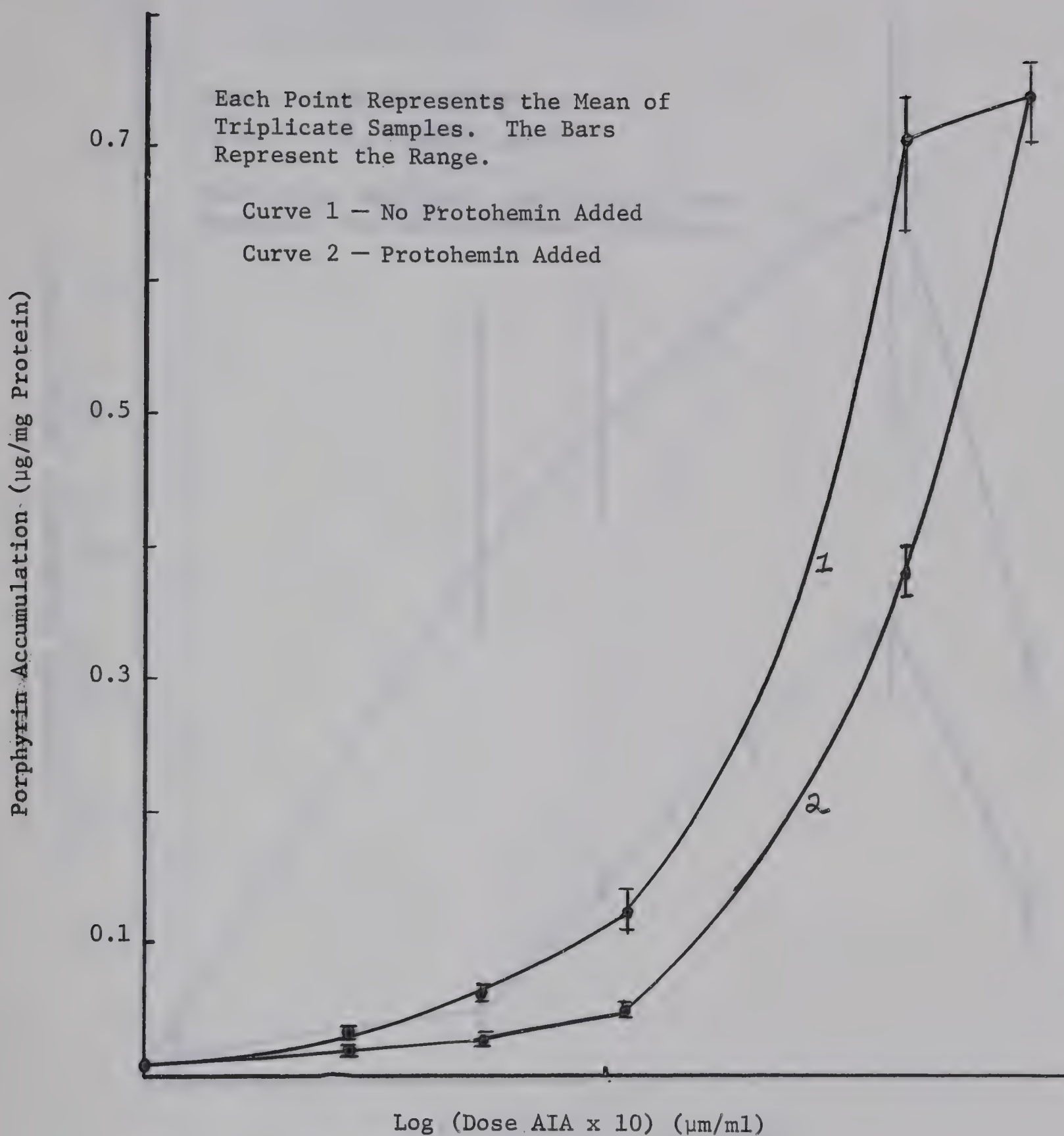


Fig. 35 Dose Response Curves for AIA in the Presence and Absence of Protohemin (0.008 µm/ml).

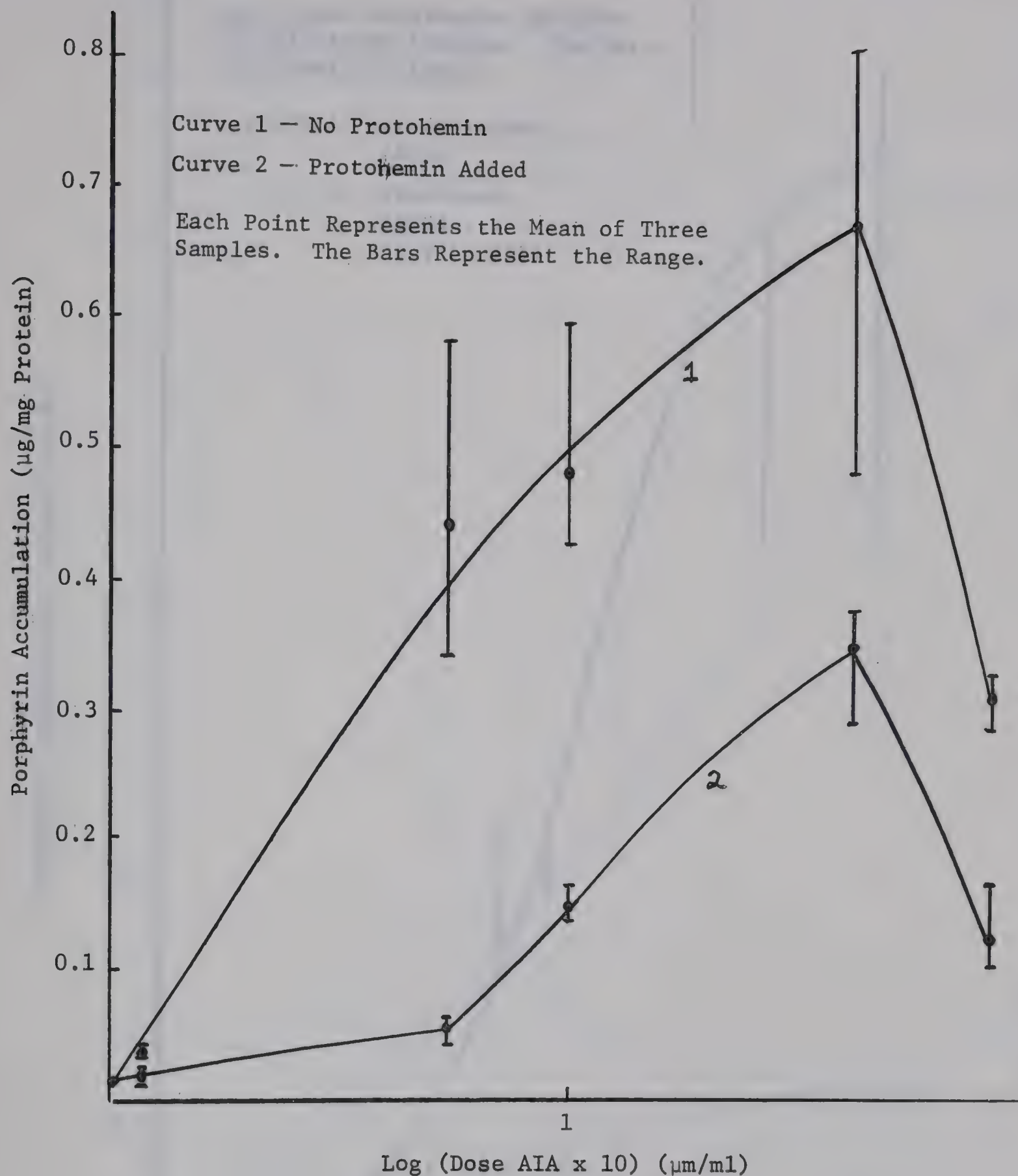


Fig. 37 Dose Response Curves for AIA in the Presence and Absence of Protohemin ($0.01 \mu\text{m}/\text{ml}$).

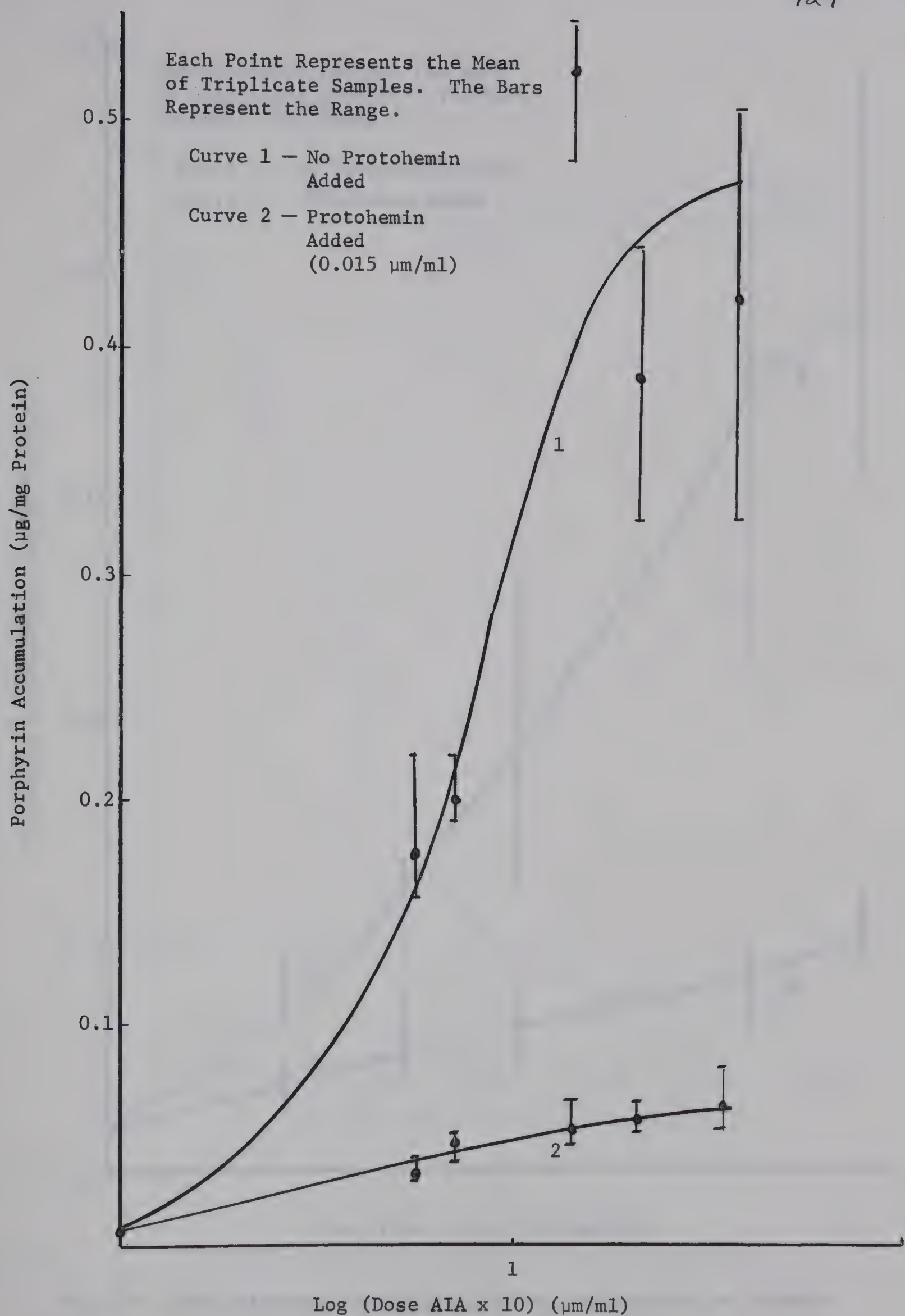


Fig. 38 Dose Response Curve for AIA in the Presence and Absence of Protohemin.

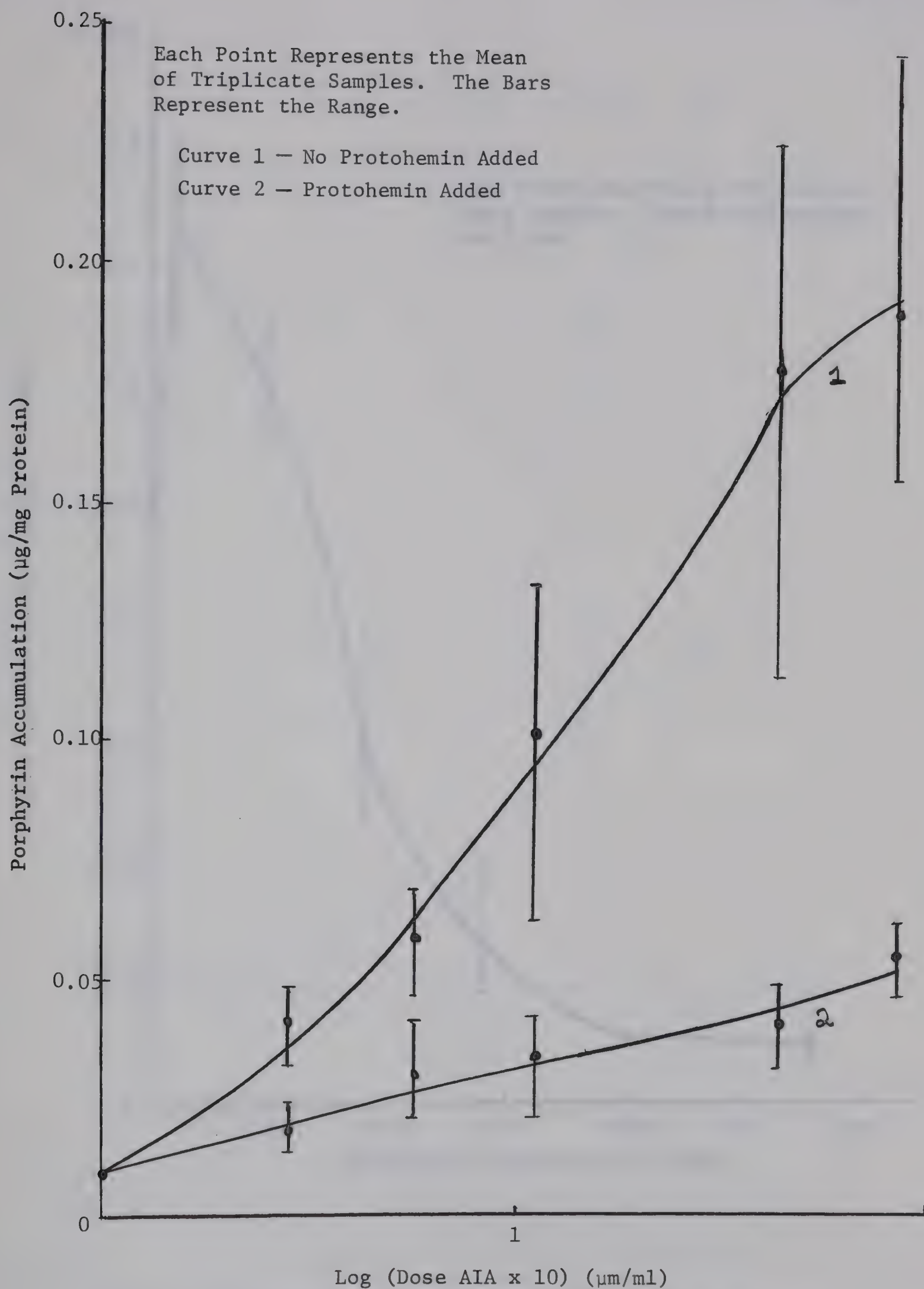


Fig. 39 Dose Response Curves for AIA in the Presence and Absence of Protohemin ($0.04 \mu\text{m}/\text{ml}$).

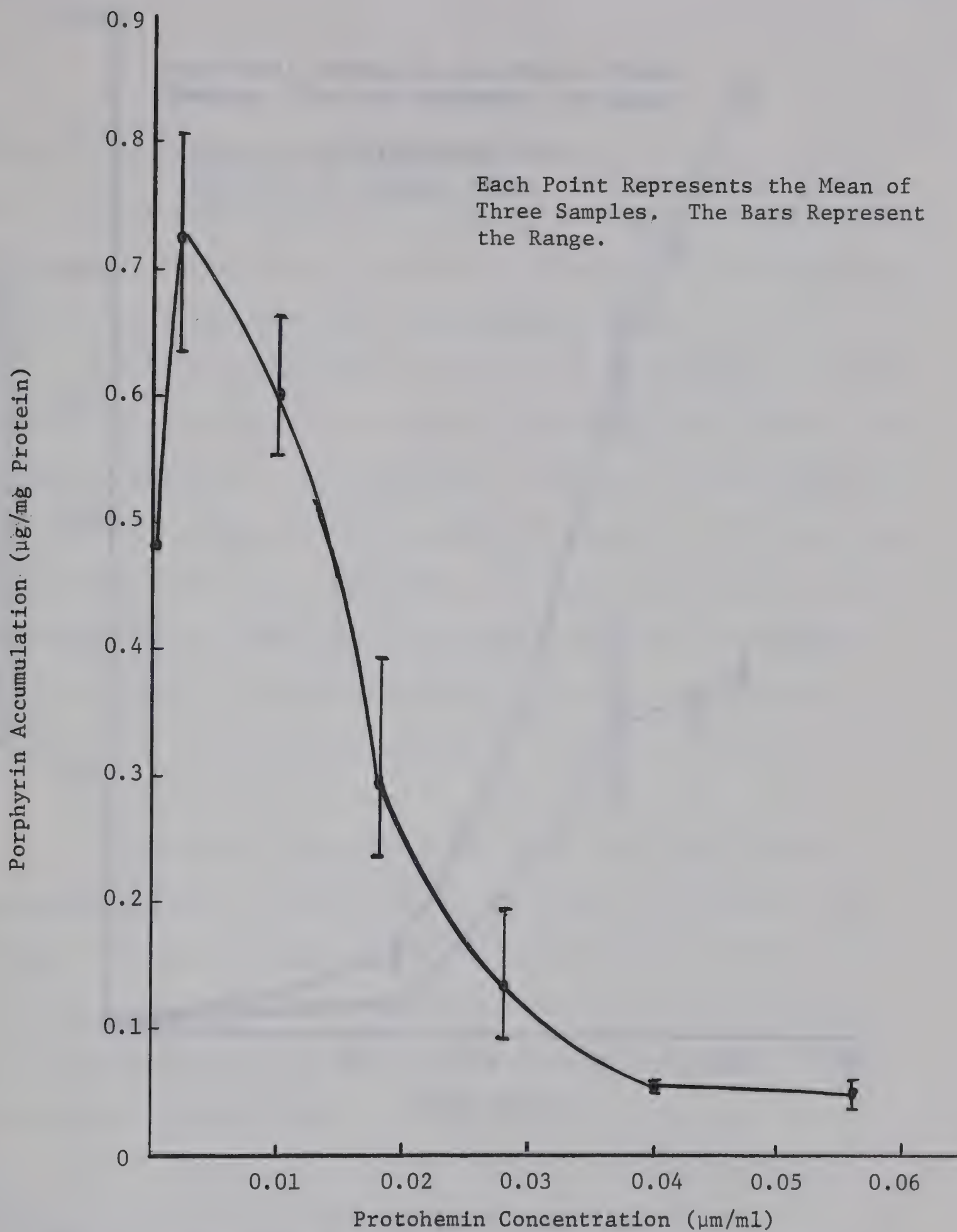


Fig. 40 Inhibitory Dose Response Curve of Protohemin on Porphyrin Accumulation by AIA (2.1 $\mu\text{m/ml}$)

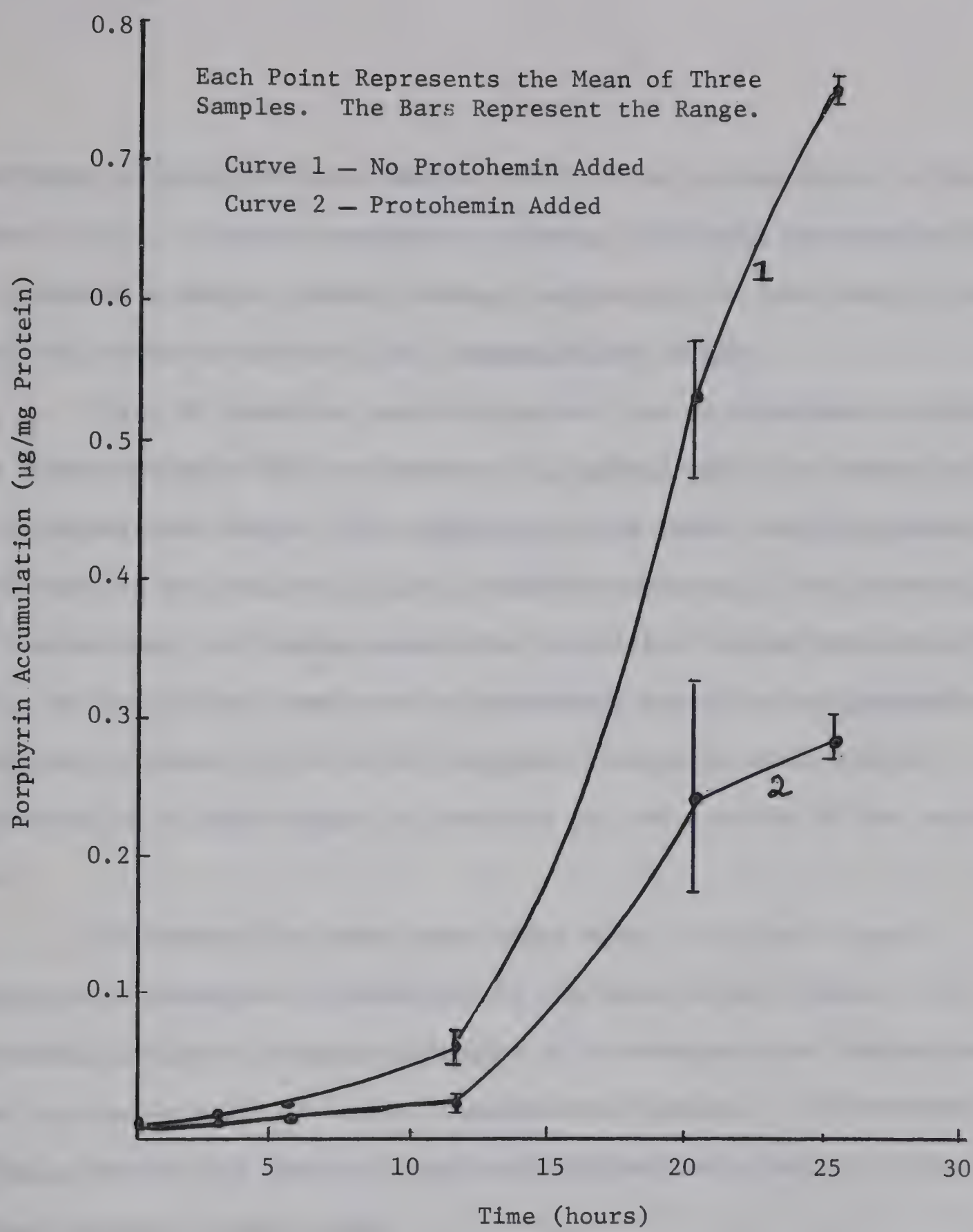


Fig. 41 The Effect of Protohemin (0.022 $\mu\text{m}/\text{ml}$) on the Time Course of Porphyrin Accumulation Following AIA (2.13 $\mu\text{m}/\text{ml}$) Administration.

inhibition of porphyrin accumulation, which is not surmountable by high doses of AIA. A further experiment employing 0.04 $\mu\text{m}/\text{ml}$ protohemin (fig. 39) produced a similar result, strongly supporting the idea that protohemin inhibits porphyria-induction in a noncompetitive manner.

Fig. 40 shows the results obtained from an experiment in which the concentration of AIA was constant (2.1 $\mu\text{m}/\text{ml}$) while the concentration of protohemin was varied. The inhibitory curve shows that high doses of protohemin do not completely block porphyria-induction. This observation is inconsistent with simple competitive inhibition between protohemin and AIA. In Fig. 41 the time course of protohemin inhibition of porphyria-induction is shown. It is noteworthy that protohemin inhibition is detectable at an early stage and persists for the duration of the experiment.

In summary the above experiments show: (1) Drug induced porphyria is sensitive to inhibition by low doses of protohemin. (2) Protohemin inhibits porphyria-induction in a noncompetitive fashion and that the simple model of Granick requires modification. (3) Protohemin markedly reduces but does not completely abolish the capacity of AIA to induce porphyrin biosynthesis.

D. Nature of Protohemin Inhibition of Porphyria-Induction

Introduction

According to present theory (Fig. 7) of control of protoheme biosynthesis, protoheme inhibits only the transcription of the structural gene for δ -ALA synthetase. Since this idea requires confirmation we planned experiments to determine if protohemin inhibition of porphyria-induction is

confined to transcription.

A method to obtain this information was suggested to us by the following studies. Peterkofsky & Tomkins (1968) studied the mechanism by which the glucocorticoid dexamethasone induced tyrosine aminotransferase (TAT) in cultured HTC cells, an established line of cells derived from Morris rat hepatoma 7288C. Cells were suspended in culture medium and experiments were carried out in two steps. The cells were first preincubated for 1.5-2.5 hours in media containing dexamethasone, cycloheximide, and actinomycin D in different combinations (Fig. 42). After washing, the cells were reincubated for 3-5 hours in media containing dexamethasone, cycloheximide, actinomycin D, or no drugs at all. At various time intervals TAT activity was determined.

Preincubation of cells with a combination of dexamethasone and cycloheximide led to a rapid increase in TAT activity upon reincubation in the absence of these reagents (Fig. 42A, curve 1). When cells had been preincubated with cycloheximide alone and then reincubated in the presence of dexamethasone, a two hour lag period in the kinetics of TAT induction was noted (Fig. 42A, curve 2). Preincubation with cycloheximide alone resulted in a negligible rise in TAT activity (Fig. 42A, curve 3). These results were interpreted by Peterkofsky & Tomkins as indicating that an intermediate required for TAT synthesis had accumulated during the preincubation period under the influence of dexamethasone even though protein synthesis was blocked by cycloheximide. If actinomycin D (Fig. 42B, curve 1) was added during the preincubation with dexamethasone and cycloheximide the subsequent rise in TAT activity was greatly reduced. Addition of actinomycin D after a preincubation with dexamethasone and cycloheximide

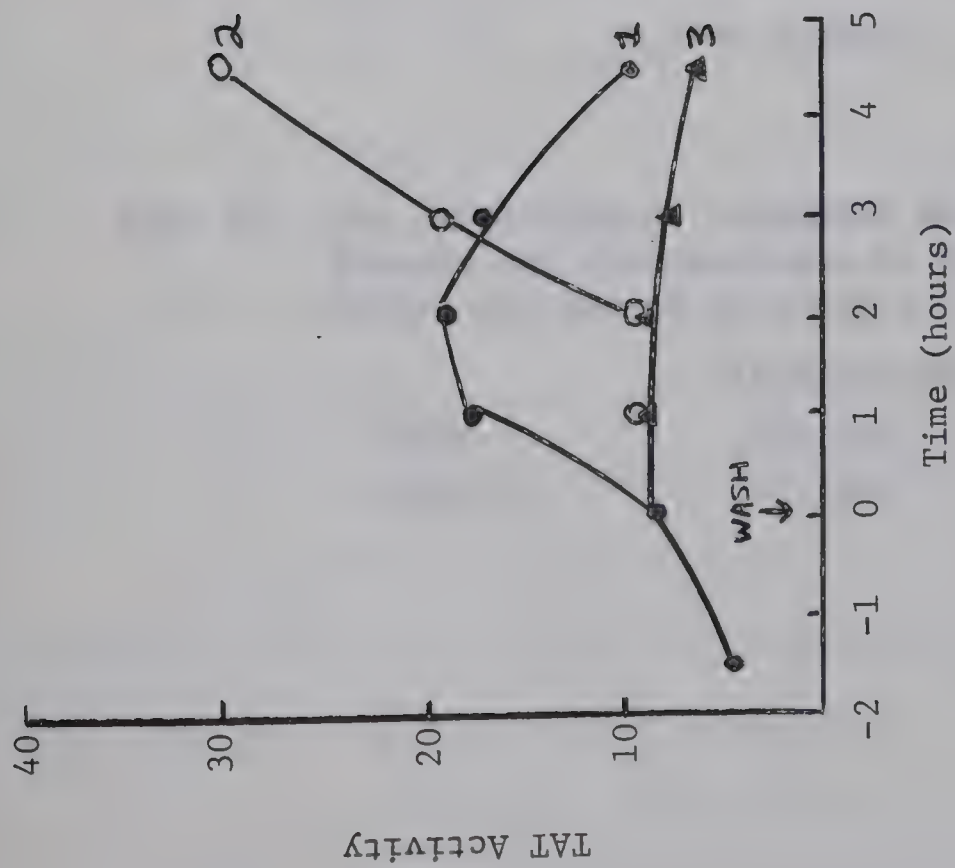


Fig. 42A TAT Activity after Preincubation of HTC Cells with or Without Various Additions of Dexamethasone and Cycloheximide (Peterkofsky & Tomkins, 1968).

Curve	Preincubation	Reincubation
Curve 1	CH + Dex	No Addition
Curve 2	CH	Dex
Curve 3	CH	No Addition

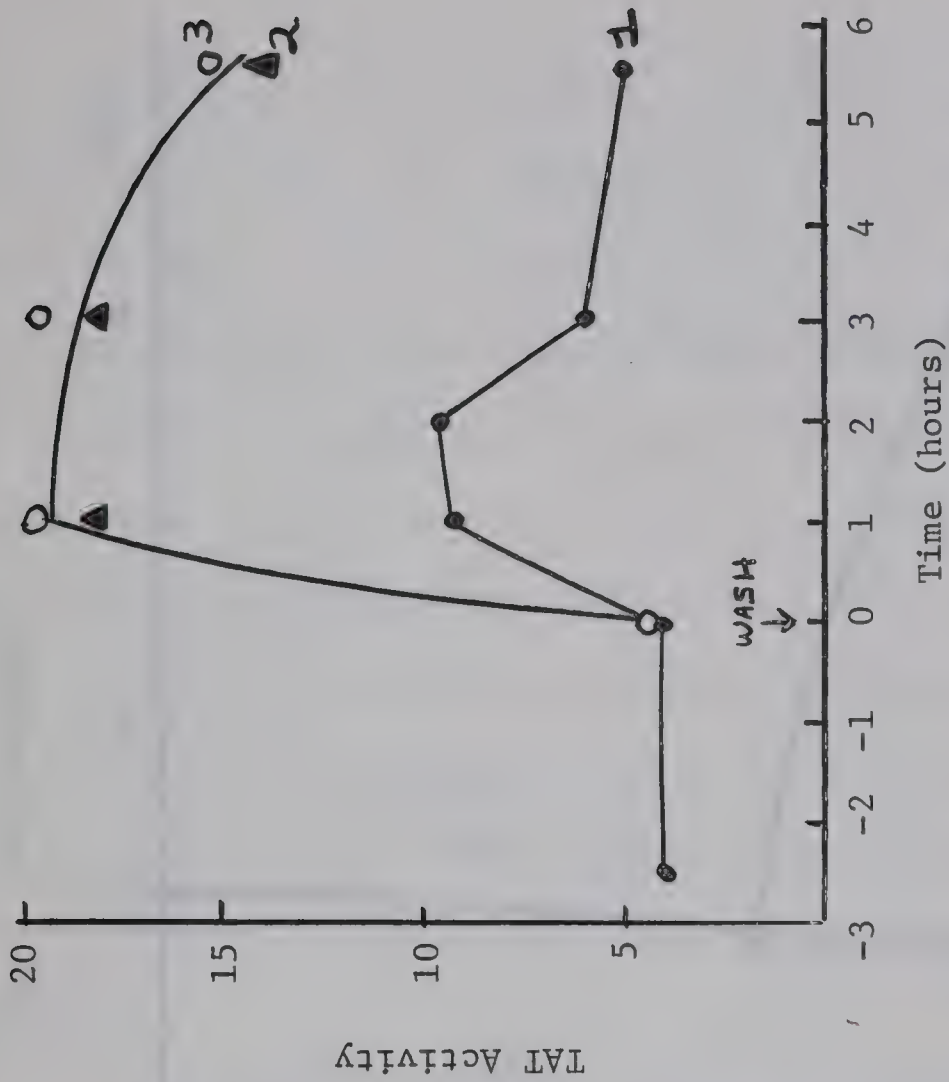


Fig. 42B The Effect of Act D Added during Preincubation on Subsequent Induction of TAT (Peterkofsky & Tomkins, 1968).

Curve	Preincubation	Reincubation
Curve 1	Act D, CH, Dex	No Addition
Curve 2	Ch, Dex	No Addition
Curve 3	Ch, Dex	Act D

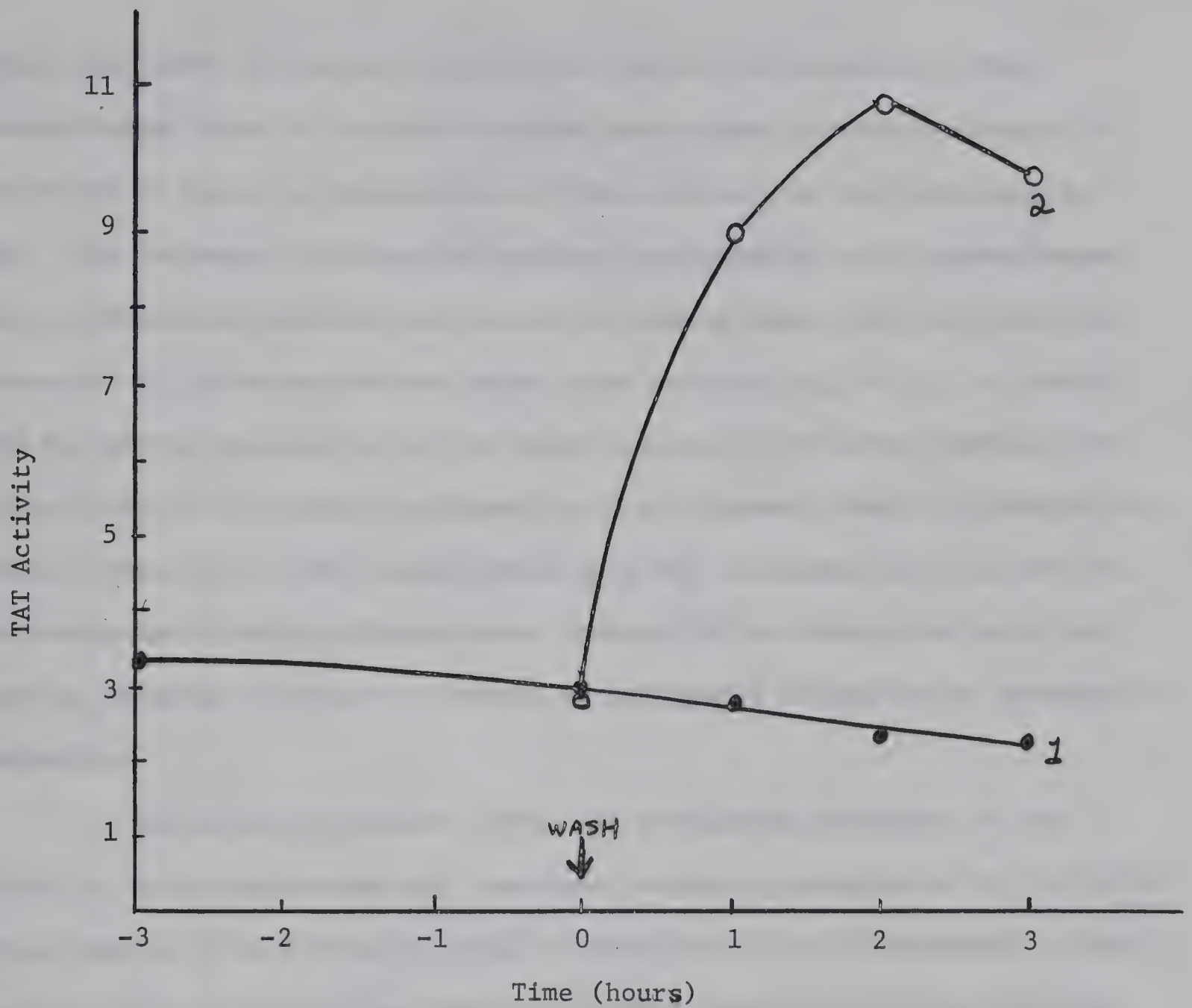


Fig. 43 The Inhibition of Induction after Preincubation with Steroid and Cycloheximide by Readdition of Cycloheximide During the Second Incubation (Peterkofsky & Tomkins, 1968).

	Preincubation	Reincubation
Curve 1	CH, Dex	CH
Curve 2	CH, Dex	No Addition

(Fig. 42B, curve 3) did not inhibit the rise of TAT activity. Thus dexamethasone acts in the preincubation period when protein synthesis is inhibited to cause an accumulation of RNA required for the synthesis of TAT. The increase in TAT activity after preincubation with dexamethasone and cycloheximide requires new protein synthesis since the rise could be prevented if cycloheximide was added after washing (Fig. 43). In view of the successful application by the above authors of different combinations of an inhibitor of protein synthesis at the ribosomal level (cycloheximide) and an inhibitor of DNA transcription to m-RNA (actinomycin D) in defining the mechanism by which dexamethasone induces TAT we thought we could use similar methods to define the nature of protohemin inhibition of porphyrin-induction.

According to Granick (1966) AIA stimulates synthesis of the m-RNA for δ -ALA synthetase and therefore porphyrin accumulates in the cells. After removal of AIA from the cells a significant rise in porphyrin accumulation (Fig. 44) takes place which is however considerably less than the rise that would have occurred in the continued presence of the drug (Granick, 1966). This suggested that the half-lives of δ -ALA synthetase and its m-RNA are measured in hours. Subsequently Granick (1966) estimated the half-life of δ -ALA synthetase to be 4-6 hours while Marver et al. (1966) estimated the half-life of m-RNA for δ -ALA synthetase in rat liver to be 2 hours.

If Granick's interpretation of the data is correct the following consequences are to be expected if various drugs are added following removal of the porphyria-inducing drug by washing:

1. Actinomycin D, a specific inhibitor of DNA transcription to m-RNA, if added during the reincubation period should not affect

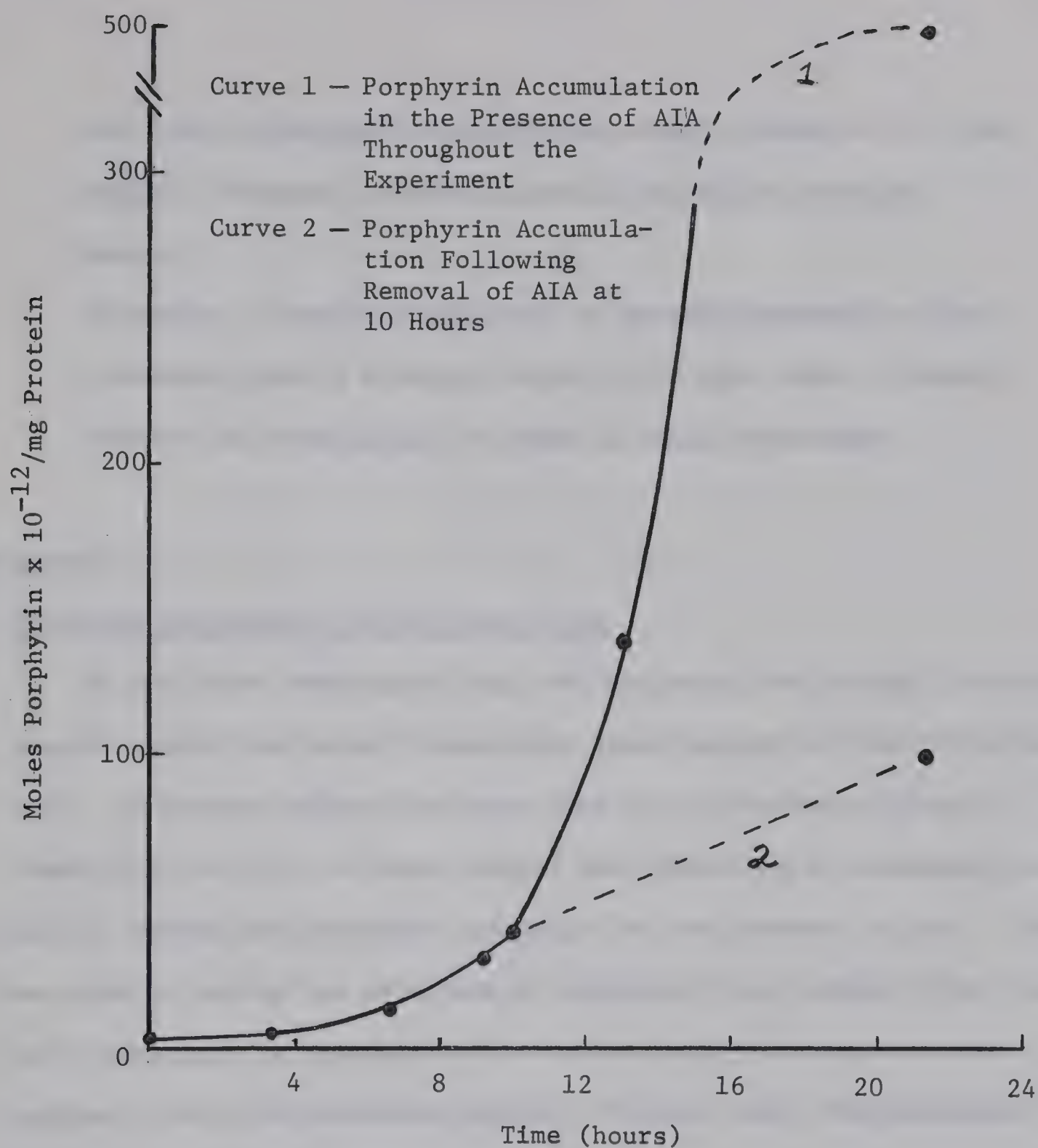


Fig. 44 Rate of Porphyrin Formation in Chick Embryo Liver Cells after Induction with AIA (0.3 mg) and after Removal of AIA (Granick, 1966).

the rise in porphyrin accumulation normally observed in this period. Moreover protohemin should act in an identical manner.

2. Puromycin, a specific inhibitor of protein synthesis at the ribosomes, should strongly inhibit this rise since it would prevent the translation of m-RNA to δ -ALA synthetase.

Experimental

i. Procedure for Removal of AIA from Cells

In the above experiment (Fig. 44) following the initial incubation Granick washed the cells 3 times with fresh medium at 5 minute intervals. He assumed without evidence that this procedure sufficed to remove all the AIA. We have checked this procedure by examining the initial medium and subsequent washings for the presence of AIA. This was done by adding the solutions to untreated chick embryo liver cells and estimating the presence of AIA by the porphyrin accumulation induced after a reincubation period. The data from this experiment are shown in table XVI.

ii. Demonstration of Porphyrin Accumulation after Removal of AIA from Cells

- a. Chick embryo liver cells were grown as monolayers on 12 petri dishes for 24 hours in the usual manner and then handled in groups of 3 in the following way:

- (1) fresh medium was added to cells and allowed to incubate for 12 hours.

- (2) fresh medium containing AIA (300 μ g/ml) was added and the

cells incubated for 8 hours.

- (3) fresh medium containing AIA (300 $\mu\text{g/ml}$) was added and the cells incubated for 12 hours.
- (4) fresh medium containing AIA (300 $\mu\text{g/ml}$) was added and the cells incubated for 8 hours after which the cells were washed once with fresh medium and reincubated for 4 hours.

At the conclusion of these time periods porphyrin content of cells and medium was estimated. The data from this experiment are shown in table XVII.

- b. Chick embryo liver cells were grown as monolayers on petri dishes for 24 hours in the usual manner and then handled in groups of 3 in the following way:

- (1) fresh medium was added to cells and allowed to incubate 19 hours.
- (2) fresh medium containing AIA (300 $\mu\text{g/ml}$) was added to 12 petri dishes and 3 dishes were incubated 19 hours, 3 were incubated 16 hours, 3 incubated 14 hours, and 3 incubated 12 hours.
- (3) fresh medium containing AIA (300 $\mu\text{g/ml}$) was added to 9 petri dishes and incubated 12 hours after which the cells were washed once with fresh medium and 3 dishes were reincubated 7 hours, 3 reincubated 4 hours, and 3 reincubated for 2 hours.

At the conclusion of these time periods the porphyrin content of cells and medium was estimated. The data from this experiment are shown in table XVIII. For convenience the data have been

utilized to plot Fig. 45.

iii. The Effect of Protohemin on Porphyrin Accumulation Following AIA Removal from Chick Embryo Liver Cells

This experiment was carried out in a similar manner to that described in ii, a & b above with the modification that protohemin was added to several petri dishes in the amounts and times indicated in table XIX.

iv. The Effects of Protohemin, Puromycin, and Actinomycin D on Porphyrin Accumulation Following Removal of AIA from Chick Embryo Liver Cells

This experiment was carried out in a similar manner to that described in iii above with the modification that puromycin and actinomycin D were added to several petri dishes in the amounts and times indicated in Table XX.

v. The Effect of Actinomycin D on Porphyria-Induction by AIA

This experiment was carried out in a similar manner to those described above with the modifications that AIA and actinomycin D were added simultaneously and no washings were done. The results are shown in table XXI.

Results and Discussion

The results in table XVI show that the medium removed from the cells treated with AIA for 8 hours has greater porphyria-inducing activity than could be accounted for by the AIA alone. This suggests that a factor derived from the cells has been released into the medium and causes potentiation of porphyria-induction by AIA. The medium derived from a single washing of AIA treated cells has only weak porphyria-inducing activity

TABLE XVI DETECTION OF AIA IN THE MEDIA AND WASHINGS OF CHICK EMBRYO
LIVER CELLS BY MEANS OF A BIOASSAY PROCEDURE.*

Solution Added to Chick Embryo Liver Cells	Duration of Treatment (hours)	Total Porphyrin Accumulation ($\mu\text{g}/\text{mg}$ Protein)
Normal Medium	16	0.006 0.006 0.007
Normal Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	12	0.065 0.066 0.052
Medium from Cells Treated with AIA (300 $\mu\text{g}/\text{ml}$) for 8 hours	12	0.095 0.090 0.095
First Washing	12	0.011 0.019 0.013
Second Washing	16	0.009 0.006
Third Washing	16	0.005 0.005 0.005

* Monolayer cultures of liver cells were grown on petri dishes as previously described. After 24 hours of growth AIA (300 $\mu\text{g}/\text{ml}$) was added to one half of the cultures while the other half received none. Following an incubation period of 8 hours the media from AIA treated cells was removed for testing. The cells were washed at 10 minute intervals with fresh media and these washings were removed for testing. The original media and washings were added individually to fresh monolayers of chick embryo cells which had been allowed to grow 24 hours and had had their media removed. These cells were then incubated a further 12-16 hours after which the porphyrin content of media and cells was determined. Each solution was tested in three separate petri dishes.

TABLE XVII PORPHYRIN ACCUMULATION AFTER REMOVING AIA FROM CHICK EMBRYO
LIVER CELLS.*

Solution Added to Chick Embryo Liver Cells	Duration of Treatment (hours)	Total Porphyrin Accumulation ($\mu\text{g}/\text{mg}$ Protein)
Normal Medium	12	0.005 0.006 0.005
Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	8	0.015 0.015 0.019
Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	12	0.034 0.030 0.028
i. Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	8	0.028 [†] 0.020
ii. Fresh Medium	0.17	0.024
iii. Fresh Medium	4	

* Monolayer cultures of liver cells were grown on petri dishes as previously described. After 24 hours of growth the cells were treated as described in the table and the porphyrin content of cells and media was determined. Each solution was tested in three separate petri dishes.

[†] For these cells the porphyrin content of the media after 8 hours of growth in the presence of AIA was determined and added to that of the wash, media, and cells following a further 4 hours of incubation.

TABLE XVIII TIME COURSE OF PORPHYRIN ACCUMULATION FOLLOWING REMOVAL OF
AIA FROM CHICK EMBRYO LIVER CELLS.*

Solution Added to Chick Embryo Liver Cells	Duration of Treatment (hours)	Total Porphyrin Accumulation ($\mu\text{g}/\text{mg}$ Protein)
Normal Medium	19	0.008 0.009 0.008
Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	19	0.328 0.298 0.373
Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	16	0.111 0.122 0.184
Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	14	0.085 0.050 0.068
Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	12	0.036 0.037 0.032
i. Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	12	0.079 [†] 0.058 0.083
ii. Fresh Medium	0.17	
iii. Fresh Medium	7	
i. Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	12	0.081 [†] 0.088 0.048
ii. Fresh Medium	0.17	
iii. Fresh Medium	4	
i. Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	12	0.047 [†] 0.077 0.050
ii. Fresh Medium	0.17	
iii. Fresh Medium	2	

* Monolayer cultures of liver cells were grown on petri dishes as previously described. After 24 hours of growth the cells were treated as described in the table and the porphyrin content of cells and media was determined. Each solution was tested in three separate petri dishes.

[†] For these cells the porphyrin content of the media after 12 hours of growth in the presence of AIA was determined and added to that of the wash, media, and cells following the indicated times of reincubation.

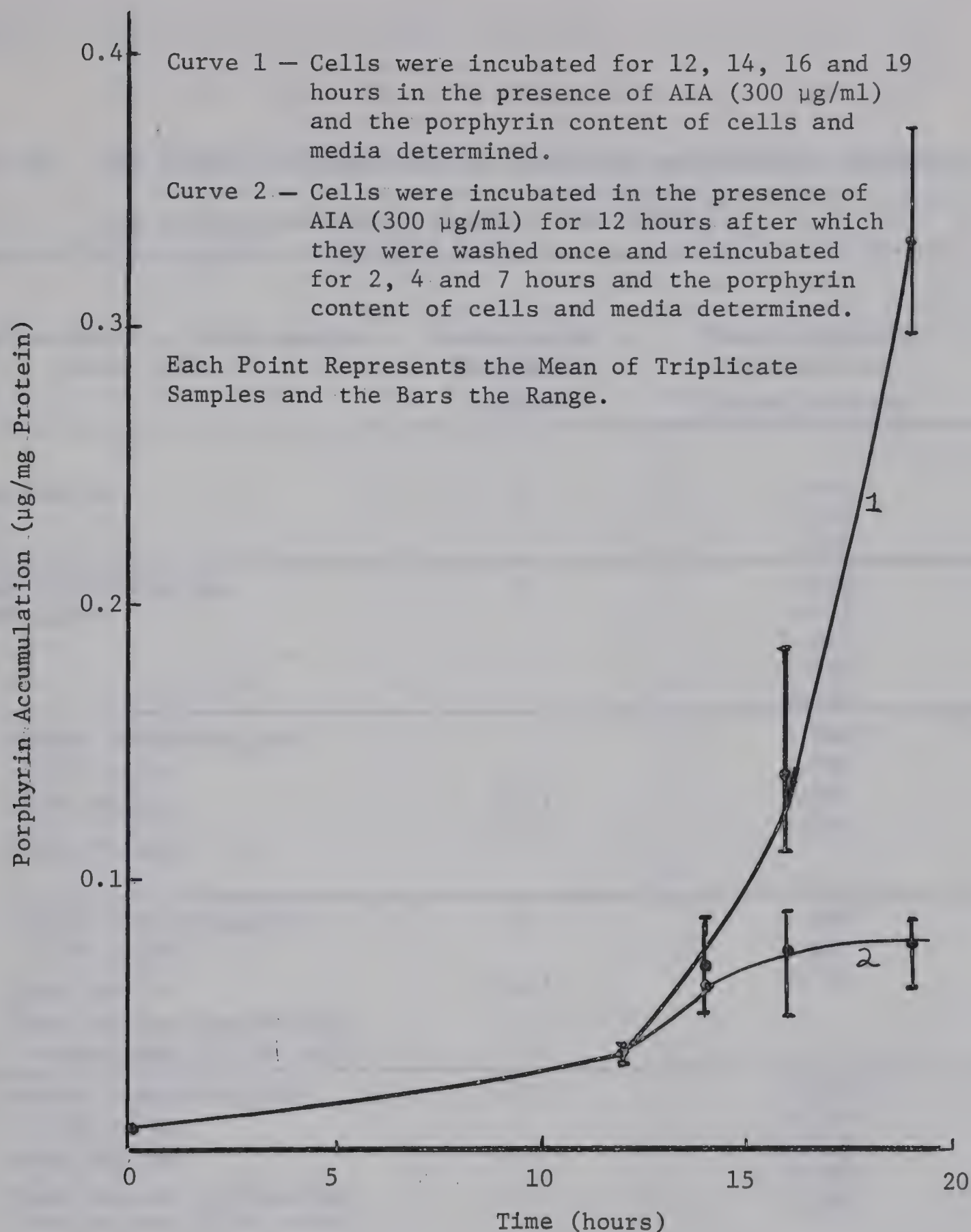


Fig. 45 Time Course of Porphyrin Accumulation Following Removal of AIA from Chick Embryo Liver Cells.

TABLE XIX THE EFFECT OF PROTOHEMIN ON PORPHYRIN ACCUMULATION FOLLOWING
AIA REMOVAL FROM CHICK EMBRYO LIVER CELLS.*

Solution Added to Chick Embryo Liver Cells	Duration of Treatment (hours)	Total Porphyrin Accumulation ($\mu\text{g}/\text{mg}$ Protein)
Normal Medium	16	0.005 0.006 0.005
Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	12	0.022 0.022 0.023 0.026 0.023
i. Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	12	0.044 [†] 0.026
ii. Fresh Medium	0.17	0.031
iii. Fresh Medium	4	0.036
i. Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	12	0.029 [†] 0.026
ii. Fresh Medium	0.17	0.028
iii. Fresh Medium Containing Protohemin (0.016 $\mu\text{m}/\text{ml}$)	4	
i. Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	12	0.019 [†] 0.019
ii. Fresh Medium	0.17	0.019
iii. Fresh Medium Containing Protohemin (0.04 $\mu\text{m}/\text{ml}$)		0.020 0.020

* Monolayer cultures of liver cells were grown on petri dishes as previously described. After 24 hours of growth the cells were treated as described in the table and the porphyrin content of cells and media was determined. Each solution was tested in 3-5 separate petri dishes.

[†] For these cells the porphyrin content of the media after 12 hours of growth in the presence of AIA was determined and added to that of the wash, media, and cells following a further 4 hours of incubation.

TABLE XX. THE EFFECTS OF PROTOHEMIN, PUROMYCIN, AND ACTINOMYCIN D ON
PORPHYRIN ACCUMULATION FOLLOWING REMOVAL OF AIA FROM CHICK
EMBRYO LIVER CELLS.

Solution Added to Chick Embryo Liver Cells	Duration of Treatment (hours)	Total Porphyrin Accumulation ($\mu\text{g}/\text{mg}$ Protein)	
		Individual Values	Mean and Standard Error of the Mean
Normal Medium	16	0.006 0.005 0.007 0.008 0.005	0.006 $\pm$ 0.0005
Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	12	0.024 0.023 0.030 0.026	0.026 $\pm$ 0.002
i. Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	12	0.044 [†] 0.040	0.045 $\pm$ 0.003
ii. Fresh Medium	0.17	0.056 0.047	
iii. Fresh Medium	4	0.038	
i. Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	12	0.039 [†] 0.023	0.028 $\pm$ 0.003
ii. Fresh Medium	0.17	0.025	
iii. Medium Containing Protohemin (0.033 $\mu\text{m}/\text{ml}$)	4	0.029 0.026	
i. Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	12	0.025 [†] 0.026	0.026 $\pm$ 0.002
ii. Fresh Medium	0.17	0.025	
iii. Medium Containing Puromycin (10 $\mu\text{g}/\text{ml}$)	4	0.023 0.034	
i. Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	12	0.056 [†] 0.041	0.046 $\pm$ 0.003
ii. Fresh Medium	0.17	0.051	
iii. Medium Containing Actinomycin D (2 $\mu\text{g}/\text{ml}$)	4	0.038 0.044	

[†] See Footnote Table XIX.

TABLE XXI THE EFFECT OF ACTINOMYCIN D ON PORPHYRIA-INDUCTION BY AIA.*

Solution Added to Chick Embryo Liver Cells	Duration of Treatment (hours)	Total Porphyrin Accumulation ($\mu\text{g}/\text{mg}$ Protein)
Normal Medium	16	0.007 0.007 0.006
Medium Containing AIA (300 $\mu\text{g}/\text{ml}$)	16	0.101 0.140 0.152 0.094
Medium Containing AIA (300 $\mu\text{g}/\text{ml}$) and Actinomycin D (1 $\mu\text{g}/\text{ml}$)	16	0.019 0.015 0.015 0.016
Medium Containing AIA (300 $\mu\text{g}/\text{ml}$) and Actinomycin D (2 $\mu\text{g}/\text{ml}$)	16	0.013 0.014 0.012 0.010

* Monolayer colonies of liver cells were grown on petri dishes as previously described. After 24 hours of growth the cells were treated as described in the table and the porphyrin content of cells and media was determined. Each solution was tested in 3-4 separate petri dishes.

whereas media derived from second and third washings have no activity. Thus one washing of AIA treated cells sufficed to remove all the AIA that could be removed by washing. For this reason in all subsequent experiments only one washing of the cells was carried out when it was desired to remove AIA. This experiment does not rule out the possibility of a small amount of "bound" AIA remaining in the cells after washing. However, subsequent experiments described below indicated that if such a fraction remained it could not account for further porphyrin accumulation.

After adding AIA to cells porphyrin accumulation was detected at 10 hours (Fig. 41, curve 1; Fig. 45) and then increased logarithmically over the next 15 hours. If AIA was removed from cells after 8 hours of incubation and the cells allowed to incubate for a further 4 hours a small but significant rise in porphyrin accumulation took place (table XVII). The time course of porphyrin accumulation following AIA removal after 12 hours of incubation is shown in Fig. 45, curve 2. Maximum porphyrin accumulation was noted 4 hours after washing, and this time interval was used in subsequent experiments. The fact that the maximum rise occurred in four hours after washing effectively rules out the possibility that porphyrin accumulation in the reincubation period is due to residual AIA in the cells. This follows from the fact that following addition of a large amount of AIA to cells there is a barely detectable porphyrin accumulation following 4 hours of incubation.

When actinomycin D at a concentration of 1 or 2 $\mu\text{g/ml}$ was added to cells simultaneously with AIA (300 $\mu\text{g/ml}$) very little porphyrin accumulation could be detected 16 hours later (table XXI). This observation which agrees with that of Granick (1966) suggests that porphyria-induction

by AIA requires new m-RNA synthesis. On the other hand when actinomycin D (table XX) was added after removal of AIA from cells it had no effect on the subsequent porphyrin accumulation. This observation suggests that the continued synthesis of m-RNA is not required for the rise in porphyrin accumulation in the reincubation period. The fact that this rise is not inhibited by actinomycin D shows conclusively that it is not produced by residual AIA. Puromycin, an inhibitor of protein synthesis at the ribosomes at a concentration of 10 $\mu\text{g/ml}$, completely abolished the porphyrin accumulation in the reincubation period showing that this rise is dependent on protein synthesis (table XX). The above observations which extend the work of Granick (1966) are generally in agreement with his interpretation of the action of porphyria-inducing drugs. However, the following observations were contrary to expectation. Thus protohemin at a concentration of 0.016 $\mu\text{m/ml}$ (table XIX) reduced and at concentrations of 0.033 $\mu\text{m/ml}$ (table XIX) and 0.04 $\mu\text{m/ml}$ (table XX) completely abolished this rise in porphyrin accumulation. The fact that protohemin is as efficacious as puromycin in abolishing the rise in porphyrin accumulation following AIA removal clearly indicates that inhibition of DNA transcription is not the only mechanism by which protohemin inhibits porphyria-induction. Either inhibition of m-RNA translation at the ribosomes or a direct inhibitory effect on δ -ALA synthetase or both could account for the effects of protohemin observed in the above experiments. It is not possible to differentiate these possibilities at present. According to Granick (1966) protohemin does not inhibit the activity of δ -ALA synthetase located in guinea pig liver mitochondria. If this observation is correct then protohemin is acting by preventing the translation of m-RNA to δ -ALA synthetase.

However, Marver (private communication) has evidence that inhibition of δ -ALA synthetase by protohemin becomes apparent in purified preparations of the enzyme.

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B29935